A Dissertation for the Degree of Doctor of Philosophy

Anti-obesity effect of sulforaphane involves inhibition of HDAC8 but not Nrf2

Nrf2 비의존적인 HDAC8 활성 저해에 의한 설포라판의 항비만 효능

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Abstract

Obesity is rising worldwide leading the increase of the risks of metabolic diseases such as type 2 diabetes, hypertension, and cardiovascular diseases. The causes of obesity is not simple because various environmental factors such as diet, sedentary time, even stress influence the development of obesity and obesity-related diseases. Since, there are always “obesogenic environment” surrounding us, sustainable, and effective long-term strategies for control obesity is required. Currently, there are several interventions of obesity including lifestyle and habit modification, bariatric surgery, and pharmacotherapy with anti-obesity drugs. However, some of them have some limitations in terms of safety, and difficulty for durability. In this context, dietary foods has some benefits because their safety is proved through the historical long-term intake, and easy to maintain continuous intervention by daily intake.

Recent findings show that epigenetic alteration can be induced by several dietary food components as well as various obesogenic environmental factors. Therefore, in perspectives of epigenetics, pharmacotherapy with natural epigenetic regulators or daily intake of natural epigenetic regulators enrich dietary food can be suggested for newly strategies for prevention or treatment of obesity.

Sulforaphane (SFN) is a natural compound enriched in daily intake available cruciferous vegetables such as broccoli sprout. There are many previous studies about the beneficial effect of SFN on various diseases including obesity and diabetes mellitus. In general, it is widely believed that these beneficial effects are

mainly attributed to the activation of nuclear factor E2-related factor 2 (Nrf2), thereby, SFN is frequently utilized as Nrf2 activating agent. However, there are some conflicting evidences about the role of Nrf2 in metabolic diseases. It indicates that it is required to investigate the role of Nrf2 in the beneficial effects of SFN on obesity and obesity-related metabolic diseases such as type 2 diabetes.

SFN newly received attention as a natural histone deacetylase (HDAC) inhibitor beyond Nrf2 mechanism especially in cancer biology. However, there is few study identifying the role of SFN as a HDAC inhibitor in obesity and related metabolic diseases. Moreover, the selectivity of SFN on HDAC families is unclear even in cancer biology.

Here, I utilized Nrf2-KO mice to determine whether the anti-obesity effect of SFN depends on Nrf2. I found that SFN suppressed high fat diet (HFD)-induced increases in body weight and improved insulin sensitivity in both Nrf2-WT and Nrf2-KO mice. These reduction was not due to alteration of food intake or lipid excretion. Therefore, to further investigate the mechanism of SFN, I examined the effect of SFN on lipid metabolism in each peripheral tissues such as white adipose tissue (WAT), liver, skeletal muscle, and brown adipose tissue (BAT) in high fat diet (HFD)-fed mice. As results, SFN induced the expression of lipases such as adipose triglyceride lipase (ATGL), and hormone sensitive lipase (HSL) in WAT resulting in the reduction of epididymal WAT weight and the increase of circulating fatty acids. SFN also promoted the consumption of fatty acids to produce energy by increasing the expression of either mitochondrial biogenesis-related proteins such as nuclear respiratory factor 1 (NRF1) in liver or mitochondrial fatty acid oxidation-related proteins such as peroxisome proliferator-activated receptor

(PPAR) families in skeletal muscle, thermogenesis-related proteins such as uncoupling protein 1 (UCP1) in BAT, which are mainly regulated by Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), a key protein in energy metabolism. For definite understanding of regulation of PGC1α by SFN, I investigated the regulatory mechanism of SFN against PGC1α. I found while there were no changes on the expression of transcription factors such as myocyte enhancer factor 2C (MEF2C), and cAMP-response element binding protein (CREB), SFN increased the transcription of PGC1α in oxidation-related organ such as liver, and skeletal muscle. However, SFN increased the global histone acetylation on histone H3 in skeletal muscle. Thereafter, I identified that SFN only selectively inhibited the HDAC8 activity rather than other classical HDAC isoforms in vitro, and there was no change on HDAC8 expression in skeletal muscle. To further examine the relevance between HDAC8 and PGC1α, I deleted HDAC8 with leti viral system in HeLa cell. I found that knockdown of HDAC8 increased the PGC1α expression, and the increase of PGC1α by SFN disappeared in shHDAC8 HeLa cell compared to shCont HeLa cells.

Overall, for the first time, I have demonstrated that SFN suppresses HFD-induced obesity regulating PGC1α-mediated lipid metabolism in several peripheral tissues by inhibiting HDAC8 activity, which is independent of Nrf2 system unlikely previous premise. These observations imply that SFN has an advantages in sustainable, and long-term intervention effect for obesity through the daily intake of broccoli or pharmacotherapy with single natural compound in perspectives of epigenetic regulation over the lifetime.

Keywords: *Obesity, Metabolic disease, SFN, Nrf2, HDACi, epigenetic regulation, lipid metabolism, PGC1α, HDAC8*

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Contents

Chapter 1

Sulforaphane-mediated prevention of metabolic diseases:

the perspective of epigenetic regulation

Abstract

Incidence of obesity is increasing worldwide as a leading cause of many metabolic diseases such as type 2 diabetes, atherosclerosis, fatty liver, and hypertension. Obesity can arise from various environmental factors, so called obesogenic environmental factors. Therefore, the interventions of obesity considering such a diversity are very important for effective control against obesity and obesity-related metabolic disease.

There are several interventions including lifestyle, habit modification, bariatric surgery, and pharmacotherapy for combating obesity. However, there are many studies supporting that epigenetic regulations such as DNA methylation, histone modification (acetylation, deacetylation, methylation) correlates the incidence of obesity, even in human subject as well as *in vitro* and *in vivo* models. Therefore, as there are recently growing recognitions that obesity is an epigenetic-related disease, it is highlighted that all options of intervention require to be applied understanding the epigenetic regulation against obesity.

Although there are several FDA-approved epigenetic regulators utilized in several diseases such as cancer, it is remained a concern about their safety. In this context, it is gradually interested in potent dietary epigenetic regulators such as sulforaphane (SFN), a natural HDAC inhibitor in intervention of epigenetic-related diseases.

Here, I reviewed the overview of obesity and obesity-related metabolic diseases such as type 2 diabetes. And I focused the significant correlation between epigenetic regulations and the development of obesity and obesity-related metabolic diseases. Additionally, especially, I suggested SFN as a natural potent

dietary agents for prevention or treatment of obesity in perspectives of epigenetic regulator.

Overall, although it is the beginning state, this review can suggest that a lot of natural potent epigenetic regulators including SFN may influence our epigenome toward resistance to obesity by applying to our daily diet.

Key words: *Epidemic obesity, obesogenic environment, metabolic diseases, obesity intervention, pharmacotherapy, epigenetic regulation, HDACi, SFN*

1.1. Introduction

Obesity has been commonly defined as a state with excess fat accumulation in the body arising from imbalance between energy intake and expenditure (1, 2). Incidence of obesity has been gradually increased worldwide in both developed and developing countries (3). Obesity is a "disease", which needs to be prevented or treated, because it is a main contributor for the metabolic diseases such as diabetes mellitus, atherosclerosis, fatty liver, and hypertension (4, 5). Not only ectopic lipid accumulation in non-adipose tissues but also various cytokines such as tumor necrosis factors (TNFα) or adipokines secreted by adipose tissue can contribute to the development of metabolic diseases (6, 7).

Since obesity is a main cause and driving factor of metabolic diseases, adequate intervention of obesity is able to control both obesity and other metabolic diseases (8). Pharmacotherapy, bariatric surgery, and lifestyle intervention are suggested as combating strategies of obesity (9-11). Especially, pharmacotherapy has received the attentions since it is relatively easy to approach. However, so far, there are only five anti-obesity drugs, which have been approved by Food and Drug Administration (FDA), including orlistat, lorcaserin, phentermine/topiramate, naltrexone/bupropion, and liraglutide (10). For decades, other anti-obesity drugs were withdrawn from the market due to their unacceptable side effects, such as heart failure and insomnia (12).

For epidemic incidence of obesity, it is gradually received attention that numerous environmental factors such as diets and lifestyle can influence epigenome to form DNA methylation and histone acetylation/methylation, thereby, modulate the obesity and the metabolic disorders over the lifetime (13, 14). Thus,

several natural compounds which have anti-obesity effects such as resveratrol, epigallocatechin gallate (EGCG), SFN are gradually focused in terms of safety compounds, and epigenetic regulators (15-19).

In this chapter, I introduce SFN as a potent preventive agent against obesity and metabolic diseases in perspective of epigenetic regulator. I covered the overview of obesity and metabolic diseases, the importance of epigenetic regulation in the development of these diseases, and the evidences supporting that epigenetic mechanism might be involved in the anti-obesity effect of SFN.

1.2. Overview of metabolic diseases

1.2.1. Causes of metabolic diseases

It is well-known that obesity is caused by imbalance between energy intake and expenditure in the body (1, 2). The "obesogenic environment" has been described as the environmental conditions promoting the onset of obesity (20). Various environment factors such as nutrition, sedentary lifestyle, and eating habits can promote obesity by complicate mechanisms (21). Once the obese state is chronically maintained, it leads to metabolic diseases including type 2 diabetes and fatty liver diseases (4, 5). Thus, it is important to understand the obesogenic environmental factors for effective intervention of obesity and obesity-related metabolic diseases.

1.2.2. Mechanistic understandings of current combating agents of obesity

Obesity and metabolic dysfunction were associated with inadequate accumulation of fat and imbalance of energy homeostasis. Therefore, the regulation of energy intake and expenditure is important strategy to control obesity and comorbidity with other metabolic disorders (22).

A century ago, energy expenditure-targeted anti-obesity drugs such as thyroid hormone preparations, and dinitrophenol were developed. These drugs increased basal metabolic rate, thereby, increase energy expenditure. Despite their high efficacy, they show adverse effects such as hyperthyroidism, overheating risk, and death (23). In next generation, most developed anti-obesity drugs targeted

energy intake. For examples, FDA approved orlistat as an only long-term treatment agent for obesity in 1999. Mechanism of orlistat is inhibition of lipid absorption from the intestine by inhibiting lipase activity as a peripheral action of regulation of energy intake (24). Several anti-obesity drugs have central action targeting hypothalamus which is a regulatory region of feeding behavior in the brain. For examples, sibutramine, a serotonin-norepinephrine reuptake inhibitor, developed in 1997 and had recognized as a representative appetite suppressant until 2010. However, it was withdrawn from drug markets due to its severe adverse effects such as nausea, cardiovascular failures, and strokes. Similarly with sibutramine, other appetite suppressant drugs such as phentermine, diethylpropion, phendimetrazine, and benzphetamine approved by FDA only for short-term use due to their adverse effects (25). The main action of recently developed drugs are also reduction of food intake increasing satiety. These drugs are approved by FDA for chronic use because they have less side effects compared to previous developed appetite suppressants. Their efficacy and safety have been proved in several clinical studies. Lorcaserin activates a selective serotonin 2C (5-HT_{2C}) receptor expressed in hypothalamus, of which mechanism is increase of satiety (26). As a combination therapy, phentermine/topiramate with phentemine (a sympathomimetic amine with anorectic effect) and topiramate (unknown mechanism), naltrexone/bupropion with naltrexone (an opioid antagonist with weight loss effect) and bupropion (an inhibitor of dopamine and norepinephrine reuptake with anorectic effect) shows lose weight effects in overweight and obese populations (27, 28). Liraglutide, a glucacon-like peptide-1 (GLP-1) receptor agonist, shows effective weight loss efficacy targeting both central nervous system

(CNS) and gut. The weight losing mechanism of liraglutide is increasing satiety and decreasing food intake by dual actions on CNS and gut (29). Anti-obesity pharmacotherapies with these developed drugs are able to play an important role in intervention of obesity with lifestyle modification and bariatric surgery for overweight and obese persons. Nevertheless, all they still needs to be applied carefully because long-term safety evaluations are not sufficient (30). In this context, recently, natural compounds have been suggested as alternative agents for controlling obesity and obesity-related metabolic diseases. Unlike synthetic drugs, these compounds such as resveratrol, EGCG, and SFN have fewer side effects, and beneficial preventive effects in chronic diseases including obesity (17-19). It has been well studied that natural compounds with anti-obesity effect also targets various mechanisms such as lipase inhibition, appetite suppression, expenditure stimulation, inhibition of adipocyte differentiation, and increase of lipolysis to reduce adipose tissue mass (31, 32). For examples, caffeine, EGCG in tea leaves showed inhibitory effect on pancreatic lipase activity, thereby, blocked the absorption of lipid in intestine (33, 34). Saponins, (−)-Hydroxycitric acid (HCA) from garcinia cambogia are natural appetite suppressants (35, 36). As a natural energy expenditure stimulants, caffeine, capsaicin, and green tea extracts are suggested (37). Moreover, there are many natural compounds to decrease adipocyte not only by inhibiting adipocyte differentiation (adipogenesis) but also by increasing lipolysis such as genistein, curcumin (38-41).

1.2.3. Suggesting mechanism for the next generation of combating agents of obesity

Considering the subsequent failure of anti-obesity drugs over the last decades, a new paradigm shift in the strategies against obesity is required for effective prevention or treatment of obesity with less safety issues. Recent studies newly have suggested that gut microbiota, circadian rhythm, and sleep can influence on obesity, type 2 diabetes, and fatty liver diseases (27, 28, 42, 43). Additionally, as many risk parameters of obesity are influenced by environmental factors, epigenetics has been received growing attentions as a novel target of anti-obesity agents beyond cancer biology (13, 29, 44, 45). In the context of epigenetic regulations, RNA interference (RNAi) such as siRNA and microRNA can also play a key role in metabolic diseases suggesting RNAi-based therapy for obesity (46). Therefore, it is also required a new insight for these newly proposed mechanisms to control complex incidence of obesity. However, it is required whether targeting these mechanisms also has good efficacy on obesity even in clinical state.

1.3. Epigenetic regulation & metabolic diseases

1.3.1. Epigenetic regulation

Chromatins are made of nucleosomes which are complex package including DNA, protein (histone and non-histone), and RNA. And histones are octamer proteins consisted of H2A, H2B, H3, and H4 for DNA packaging in nucleus contributing to nucleosome structure (47). Epigenetic regulation is the modulation of gene expression by changing DNA chromatin structure without changes on DNA own sequence. The epigenetic regulation contains the methylation on cytosine nucleotide in DNA sequence, post-translational modification of histone proteins, and non-coding RNAs like miRNA. Once the epigenetic alteration occurs on DNA or histones, accessibility of transcription factors becomes different due to change of DNA conformation. The epigenetic changes induce reversible, but heritable variations from gene expression of same DNA sequences (48, 49).

It has been shown that epigenetic abnormality induces aberrant development or certain diseases such as cancer (50). Therefore, there are many clinical evidences supporting that epigenetic regulators can have therapeutic effect on certain diseases, especially cancer (24, 25, 30, 51). Among them, 5 drugs such as decitabine, azacytidine, vorinostat, romidepsin, and belinostar were already approved by FDA with their anti-cancer effects (48). As the prevalence of obesity is increasing over the past several decades, growing attentions are placed on the role of epigenetic regulation in obesity and obesity-related metabolic diseases such as diabetes. Several metabolic genes such as peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1α), retinoic acid receptor α (RARα), and

fatty acid-binding protein 4 (FABP4) are referred to “epiobesigenic genes”, which play roles in development of obesity modulated by epigenetic regulation (52).

1.3.2. DNA methylation & metabolic diseases

The most widely documented epigenetic modification is DNA methylation. The methylation is direct addition of a methyl group ($-CH_3$) on cytosine nucleoside next to a guanine known as CpG site in DNA sequence. The methylation on CpG site is mainly mediated by methyltransferase (DNMT), which contains DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMTL. If the methyl group is transferred to CpG site in promoter of certain genes, transcription factors fail to bind to the promoter region and induces the transcriptional silence of those genes. Since inappropriate methylation on CpG site leads to diseases-associated gene expressions profile, recovering effects by DNA methylation inhibitors may be a therapeutic intervention in epigenetic-related diseases such as cancer, obesity, and so on (29).

Many previous studies demonstrated that DNA methylation correlated with obesity. For examples, it has been reported that high-intensity exercise decreases methylation on the promoter of metabolic genes such as PGC-1α, and mitochondria transcription factor A (TFAM) resulting in alleviation of obesity (29). On the other hand, different types of diets (e.g. high fat diet) cause changes in DNA methylation leading to promotion of obesity (44, 53). The group with intervention for weight loss show differences the level of genome-wide methylation compared to the control group (54, 55). Additionally, the DNA methylation in promoter of several metabolic genes can affect metabolic effects in development of obesity and

type 2 diabetes. These metabolic genes include peroxisome proliferator-activated receptor α (PPARα) related to lipid metabolism (56), leptin, melanocortin 4 receptor (MC4R), neuropeptide (NPY), and pro-opiomelanocortin (POMC) which are associated with appetite regulation (53, 57-59), interferon γ (IFNγ), and tumor necrosis factor α (TNFα) related to inflammation (60, 61), fatty acid synthase (FAS), and uncoupling protein 1 (UCP1) which are involved in lipogenesis and thermogenesis, respectively (62, 63).

However, it seems to be lacked that the consistent evidences about the consistent CpG site associated with obesity to support the strong correlation between global DNA methylation and obesity (45).

1.3.3. Histone modification & metabolic disease

Histones are major components of nucleosome structures and contain each 2 proteins of H2A, H2B, H3, and H4. Histones have positive electric charges for binding to electrically negative charged DNA. Apart from methylation, histone modification is also important epigenetic regulation to control gene transcription by compacting or relaxing chromatin to less or more accessible to transcriptional machinery. The post-translational histone modification is mainly mediated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). While HAT transfers acetyl group to lysine amino acid residues, HDAC removes acetyl group from acetyl-lysine (Ac-Lys), which make differences on electrostatic change inducing alteration of binding affinity between histone and DNA resulting in activation or repression of gene transcription (64).

Especially, HDACs play central roles in the large range of epigenetic

regulation due to their various subtypes. Classical HDACs contain total 11 types except Class Ⅲ HDACs (sirtuins), which are distinguished from other classes because their action is not major in histone modification. 11 mammalian HDACs are divided into 4 classes; class Ⅰ HDACs (HDACs 1, 2, 3, and 8), class Ⅱ HDACs (HDACs 4, 5, 6, 7, 9, and 10), classⅣHDACs (HDAC 11) as their characteristics (65).

HDACs play different roles and are expressed in different location at different extent even in the same class type. While class Ⅰ HDACs (HDACs 1, 2, 3, and 8) were ubiquitously expressed in various tissues and located in the nucleus, class Ⅱ HDACs (HDACs 4, 5, 6, 7, 9, and 10) were expressed in limited tissues and shuttled between the cytoplasm and nucleus. It is little known about ClassⅣHDAC (HDAC 11). On the other hands, ClassⅢ HDACs (SIRT 1-7) play a distinguishable role as nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent pathway. Therefore, classical HDACs have total 11 HDACs (HDAC1-11). Since the role of HDACs would be very different depending on tissues, it is necessary to consider tissue-specific roles of each HDACs (65).

Previous studies provide evidences that abnormal histone modifications are associated with epidemic disease including cancer, immune diseases, and neurodegenerative diseases (23, 42, 66). Therefore, therapies based on understanding of histone modification such as using HDAC inhibitors (HDACi) may be a good therapeutic intervention in several complex diseases associated with epigenetics such as cancer.

Some previous studies provided evidences that deacetylation by HDACs

in certain metabolic genes as well as global histone modification has relevance with metabolism (67). For examples, it was revealed that class Ⅱa HDACs (HDAC 4,5, and 7) respond to glucagon or insulin as mediators of cAMP –activated protein kinase (AMPK) pathway and regulate glucose production in liver. In detail, class Ⅱa HDACs deacetylate and activate the transcription of gluconeogenesis-related genes such as glucose 6-phosphatase (G6Pase), consequently result in hyperglycemia in diabetic mouse models (43). HDACs also play role in muscle differentiation. HDAC1 interacts with MyoD inhibiting MyoD-dependent transcription of muscle-specific genes such as muscle creatine kinase (MCK), and myosin heavy chain (MHC) in myocytes (68). On the other hand, HDAC4 and 5 which are included in class Ⅱ HDACs inhibit myocyte enhancer factor 2 (MEF2) activity in differentiating myocytes (69). Additionally, post-translational modification of histone by HDAC also affect the transcription of several metabolic genes such as brain-derived neurotrophic factor (BDNF), an depression-related neurotrophic factor gene (70), adiponectin, insulin, and glucose transporter 4 (GLUT4) relate to glucose homeostasis (71-73), and glucocorticoid receptor (GR) involved in stress mechanism (74).

1.3.4 Epigenetic regulators as potential metabolic therapeutics

Since there are several previous studies support that epigenetic regulations including histone modification modulate various metabolic pathway in different organs such as liver, muscle, it is necessary to consider epigenetic regulation in intervention of obesity and obesity-related metabolic disease. For combating obesity, epigenetic regulators as well as lifestyle modification such as physical

activity, and diet restriction can change the epigenetic mechanism in daily life.

For examples, the epigenetic regulators, compounds modulating epigenetic mechanisms, including DNMT inhibitors (DNMTi), and HDAC inhibitors (HDACi) have been developed. So far, total 5 epigenetic drugs including Vidaza (5-Azacytidine), and Dacogen (Decitabine) as DNMTi and suberanilohydroxamic acid (SAHA), Beleodaq (Belinostat), and Istodax (Romidepsin) as HDACi are approved by FDA. Moreover, other epidemic regulator candidates are continuously developed besides of these drugs. 5-Azacytidine and decitabine are nucleoside (cytidine) analogs, thereby prevent DNMT action and DNA methylation by integrating to DNA instead of nucleoside for DNA synthesis. HDACi contain several categories including hydroxamates, and cyclic peptide depending on their structures. Suberanilohydroxamic acid (SAHA), the first FDA-approved HDACi as well as as belinostat is in hydroxamate category and a pan-inhibitor targeting both class Ⅰ, and class Ⅱ HDACs. Romidepsin is in cyclic peptide category. Clinical trials are undergoing for these drugs and show good clinical results in certain diseases, especially cancer (48, 75). These drugs have been mainly focused in cancer biology. Since it has been recently reported that epigenetic mechanisms are also involved in obesity and obesity-mediated metabolic diseases, there are evidences providing that epigenetic regulators can also control obesity and obesity-mediated metabolic diseases (52).

However, there are limitations due to the fact that they are all pan-inhibitors which did not target specific CpG sites or specific HDAC isoforms. Therefore, it is necessary to further develop the inhibitors against selective HDAC isoforms for more effective prevention or therapy of epigenetic-related disease

including not only cancer but also obesity and its related diseases (76, 77).

Several studies highlighted that natural compounds as well as nutrients, and diets also have epigenetic regulatory effect by modulating DNA methylation or histone modification. These support natural epigenetic regulators could be also helpful for prevention or therapy of epigenetic-related disease such as cancer, obesity, diabetes, and so on.

For examples, a specific B vitamin such as folate, vitamin B-12 is a nutrient participated in DNA methylation process as a methyl donors (78). Therefore, deficiency of these nutrients during pregnancy influences fetal programming inducing aberrant development (79). Moreover, some of natural compounds including isoflavone such as genistein, and tea polyphenols such as epigallocatechin gallate (EGCG) also inhibit DNMT enzyme reducing DNA methylation in cancer-related genes such as retinoic acid receptor β (RARβ), and reversion-inducing cysteine-rich protein with Kazal motifs (RECK) in cancer cells (80, 81). High fat diet (HFD) can also alter DNA methylation status on CpG site in melanocortin-4 (Mc4r) genes associated with body weight regulation compared to standard diet *in vivo* (58). EGCG also enhances HDAC activity suppressing the pro-inflammatory action of nuclear factor-kappa B (NF-κB) in obese subjects (82). Curcumin degrades HAT enzyme, especially p300/CREB-binding protein (CBP) attenuating HFD-induced obesity and insulin resistance-associated abnormalities including lipogenesis, and inflammatory responses (83). Some of natural compounds including SFN from broccoli, and diallyl sulfide from garlic can inhibit HDAC enzyme and increase histone acetylation exerting anti-cancer effects (84, 85). Especially, many previous studies supported that inhibitory effect of SFN on

HDAC enzyme activity is identified even in human subjects as well as *in vitro* and *in vivo* (84, 86-89).

1.4. SFN, as an epigenetic regulator

SFN, one of isothiocyanates, is plentiful in cruciferous vegetables, especially in broccoli sprouts. It is well known that SFN has beneficial effects on numerous diseases such as anti-cancer, anti-microbial effect, anti-oxidative effect, and neuroprotective effect (3, 90-92). In general, it is widely believed that SFN can exert the beneficial effects on various diseases via activation of nuclear factor E2-related factor 2 (Nrf2) (93-97). Moreover, a lot of researches frequently have utilized SFN as a positive control or activating agent to investigate the Nrf2 pathway (98-101).

Presently, however, it is increasing the supporting evidences that SFN served as a representative example of epigenetic regulator, especially HDACi (89). Several studies demonstrated that SFN has anti-cancer effect in colon and prostate cancer cells by inhibiting HDAC activity as a distinct mechanism from previous proposed other mechanism such as Nrf2 (86, 87). The inhibitory effect of SFN on HDAC enzyme triggers the suppression of tumor growth in xenograft model and decreases in spontaneous intestinal polyps increasing global histone acetylation in colon and intestine in Apc^{min} mice model (84, 88). These observations indicate that a role of SFN as a HDAC inhibitor is available *in vivo*. Additionally, intake of SFN-rich broccoli sprouts induced inhibition of HDAC activity in peripheral blood mononuclear cells (PBMCs) of all human subjects in study (88). These findings supported that dietary intake of SFN-rich broccoli sprouts can modulate epigenetic phenomena by inhibition of HDAC in daily life, which can be expected even at the

same dose of SAHA, a clinical HDAC inhibitor approved by FDA.

Previous findings showed the HDAC inhibitory effect of SFN in various models especially in cancer. Moreover, recent evidences suggest that epidemic regulation play a key role in obesity and metabolic disease as well as cancer. Therefore, I suppose that SFN can be a potent epigenetic regulator, as a natural HDACi, in epidemic-related development of obesity and obesity-related metabolic diseases.

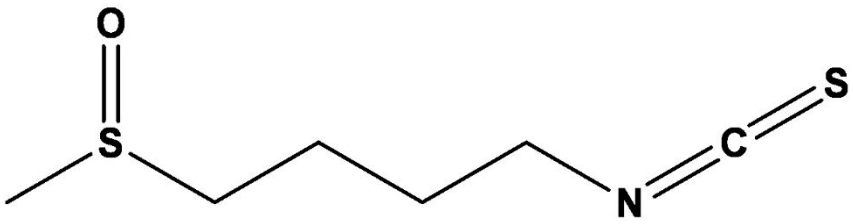

Figure 1. The structure of SFN

1.5. Conclusion

As the prevalence of obesity is globally increasing, thereby, and the risk of metabolic disease is rising, adequate intervention is needed to reduce the increasing rate of obesity. Presently, lifestyle and behavior modification, bariatric surgery, pharmacokinetics are available interventions for overweight and obese persons. However, currently approved drugs have limitations in safety issues because of their adverse effects.

As growing epidemic obesity, obesity is recognized as not just gene-driven but environment-driven diseases. Recent findings related to the relationship between epigenetic regulation and obesity implies that epigenetic regulators are expended to apply in various diseases such as obesity and obesity-related diseases, not just cancer. Therefore, epigenetic-based intervention of obesity will be helpful to respond for the influences from dynamic environmental factors. In this context, it is growing interests in the natural compounds including SFN in the field of obesity and obesity-related diseases, which have epigenetic modulation ability and relatively safe than synthetic drugs.

Here, I found that SFN suppresses high fat diet (HFD)-induced obesity promoting PGC1α transcription by specific inhibiting HDAC8 activity increasing global acetylation of histone H3 and histone H4 in skeletal muscle. These findings are distinct from Nrf2, a representative target of SFN, and interpreted in perspective of epigenetic regulation. Since there are evidences that the HDAC inhibition effect of SFN is also found even in human, SFN may be potentially strong anti-obesity agents as an epigenetic regulator, HDACi. Moreover, epigenetic regulatory effect of SFN also can be obtained from the intake of SFN-enrich

dietary food such as broccoli as well as pharmacotherapy with the intake of SFN as a single compound, which means the beneficial effect of SFN may be easily sustainable over the lifetime.

1.6. Perspectives

Epigenetic-based therapy for obesity and obesity-related metabolic diseases are novel approaches to consider dynamic environmental factors promoting onset of obesity. Thus, this approach may let us to unknown contributors in development of obesity and obesity-related metabolic diseases. However, previous researches on epigenetic therapy have limitation in terms of non-specificity of the most epigenetic regulators. While epigenetic alteration is revealed as a hallmark of cancer, more evidences are needed to strongly confirm the relationship with epigenetic regulation and obesity. Therefore, further investigation of various natural epigenetic regulators and their precise mechanisms will extend our knowledge on the epigenetic therapies for obesity and obesity-related metabolic diseases. And it is required to investigate more for understanding complexity of epigenetic regulation in obesity and obesity-related metabolic diseases, and newly developing next generative epigenetic regulators considering personal variations.

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Chapter 2

Sulforaphane suppresses high fat diet-induced obesity and insulin resistance independent of Nrf2 system

Abstract

Previous evidences about the beneficial role of SFN underlying nuclear factor E2-related factor 2 (Nrf2) mechanism in various disease such as cancer, and neuro degenerative diseases are now in question at least in obesity and obesity-related diseases such as type 2 diabetes. It has been reported that SFN has the anti-obesity effects *in vitro* and *in vitro*. However, since conflicting results on the role of Nrf2 in obesity between the researches with Nrf2 activator such as 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole (CDDO-imidazole) and Nrf2 loss-of function studies were reported, it is now necessary to review the role of Nrf2 in the anti-obesity effect of SFN to understand exact mechanisms.

Herein, I investigated whether the anti-obesity effect of SFN is dependent on Nrf2 or not using Nrf2-wild type mice and Nrf2-knockdown mice model. As results, I found that SFN attenuated high fat diet (HFD)-induced body weight gaining in both Nrf2-WT and Nrf2-KO mice. Moreover, SFN also improved glucose tolerance and insulin sensitivity in both mouse genotypes, which was referred from the results of OGTT, ITT, and HOMA-IR index. I also found that this beneficial effects of SFN were not attributed to the reduction of food intake (kcal/day) or the increase of excretion of fecal lipids (mg/feces g dry weight). Collectively, I revealed that the beneficial effects of SFN on obesity and insulin resistance were still available in absence of Nrf2. This implies that another mechanism may be involved in SFN's action in HFD-fed mice.

Key words: *Sulforaphane (SFN), Nrf2, obesity, insulin resistance*

2.1. Introduction

Recent reports have demonstrated that sulforaphane (SFN) alleviates obesity by inhibiting adipogenesis and promoting lipolysis (1-4). The transcription factor nuclear factor (erythroid-derived 2)-like 2, also known as Nrf2, is a well-known target protein of SFN and has been noted to regulate lipid metabolism (5, 6). For example, Whitman et al. reported that Nrf2 modulated metabolic properties of skeletal muscle in streptozotocin-induced diabetic atrophy (7). Heme oxygenase-1, via Nrf2-mediataed transcription control, was shown to regulate cardiac mitochondrial biogenesis (8). Proteomic analysis of Nrf2 deficient transgenic mice revealed that Nrf2 plays an important role in the synthesis and metabolism of fatty acids and other lipids in the liver (5). The enzyme ATP-citrate lyase, responsible for acetyl-CoA production, was negatively regulated by Nrf2, suggesting that Nrf2 might be a major regulator of cellular lipid disposition in the liver (5). NAD(P)H:quinone oxidoreductase 1, one of the major Nrf2-regulated proteins, was shown to reduce hypertrophy in 3T3-L1 adipocytes (9). Synthetic Nrf2 activators such as 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole (CDDO-imidazole) and oltipraz, as well as several natural compounds activating Nrf2 such as carnosic acid, carnosol, and curcumin have been also shown to have anti-obesity effect *in vitro* and *in vivo* (5, 10-13). Therefore, it seems possible to assume that Nrf2 might be a molecular target of SFN to inhibit adipogenesis and promote lipolysis.

Although there are plentiful evidences to showing that Nrf2 activators can be developed as potent anti-obesity drugs, recent studies using genetically modified

Nrf2 knockout (KO) mouse model supports other role of Nrf2 in lipid metabolism (14-16). For example, deficiency in Nrf2 resulted in impaired adipogenesis and protected against diet-induced obesity (14). Nrf2-KO mice were partially protected from high fat diet (HFD)-induced obesity and developed a less insulin-resistant phenotype (16). Nrf2 deficiency improved glucose tolerance in mice fed a HFD (15). Nrf2 promoted the major proteins of adipogenesis such as CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) by direct binding (3, 14, 17, 18). Moreover, Keap1-knockdown mice, which exhibit enhanced Nrf2 activity, increased markers of metabolic syndrome after long-term HFD feeding (19). Considering these conflicting evidences on the role of Nrf2 in lipid metabolism, a precarious assumption that Nrf2 might be a molecular target of SFN to inhibit adipogenesis and promote lipolysis needs to be re-viewed and clearly studied.

Therefore, I investigated the role of Nrf2 in the effect of SFN on HFD-fed Nrf2-wild type (WT) and Nrf2-KO mice. I found that the anti-obesity effects of SFN was shown in both HFD-fed Nrf2-WT and Nrf2-KO mice, suggesting that Nrf2, the well-known target proteins of SFN, was not involved in SFN-mediated anti-obesity effects. Moreover, I found that SFN improves insulin sensitivity in both HFD-fed Nrf2-WT and Nrf2-KO mice. These observations suggest that other mechanism than Nrf2 attributes to the anti-obesity effects of SFN. It is noteworthy that Nrf2 does not play a role in SFN-modulated metabolic physiology in HFD-fed mice.

2.2. Materials and Methods

2.2.1. Animals

C57BL/6J Nrf2-KO mice, originally developed by Prof. Masayuki Yamamoto (20), were obtained from RIKEN BRC (Tsukuba, Japan). The offsprings were genotyped as previously described (20). Primer pairs used in polymerase chain reaction (PCR) are listed in table 1. Mice were housed with free access to food and water in the animal facility of the Seoul National University in temperature-, light-, and humidity-controlled rooms with a 12-h light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-110126-8).

Table 1. The primer sequences used for genotyping

Gene	Sequence
NRF2-5' (sense for both genotype)	5'-TGGACGGGACTATTGAAGGCTG-3'
lac Z (antisense for LacZ)	5'-GCGGATTGACCGTAATGGGATAGG-3'
Nrf2-AS (antisense for WT mice)	5'-GCCGCCTTTTCAGTAGATGGAGG-3'

2.2.2. Treatment

Age-matched male Nrf2-WT and Nrf2-KO mice (6-8 weeks old) were fed a chow diet (CD) or a high fat diet (HFD) (60 kcal% Fat, Research Diets, New Brunswick, NJ, USA) for 15 weeks. Mice were divided into three groups (n=6-7 per group): (1) CD + phosphate-buffered saline (PBS) control group, (2) HFD + PBS group, and (3) HFD + SFN group. SFN (Santa Cruz Biotechnology, Santa

Cruz, CA, USA) was dissolved in PBS and orally administrated at doses of 50 mg/kg body weight every day for 15 weeks.

2.2.3. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

Oral-GTT (OGTT) was performed by glucose oral administration (10% glucose, 10 ml/kg body weight) to Nrf2-WT and Nrf2-KO mice fasted for 16 hr. Blood glucose was measured from tail with Accu-Chek Blood glucose meter (Roche Diagnostics, Indianapolis, IN, USA) at 0, 15, 30, 60, and 120 min after glucose administration.

ITT was carried out by intraperitoneal (i.p.) insulin injection (1 U/kg body weight) to Nrf2-WT and Nrf2-KO mice after fasting for 4 hr. Blood glucose was measured from tail with Accu-Chek Blood glucose meter at 0, 15, 30, 60, and 120 min after injection of insulin.

2.2.4. Serum biochemistry

Blood samples were collected from tail with heparin capillary tube (Kent Scientific, Torrington, CT, USA) after 16 hr fasting. Fasting glucose was measured using an Accu-Chek Blood glucose meter and insulin level was analyzed using mouse insulin ELISA kit (ALPCO, Salem, NH, USA). Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated by the equation of [glucose (mg/dl) × insulin (ng/ml)]/405 with substitution of fasting glucose and insulin concentration values (21).

2.2.5. Food intake

Food intake (g) per day was measured every week. Caloric intake was calculated by multiplying amount of food intake (g) by physiologic fuel value of CD or HFD, 3.85 kcal/g or 5.24 kcal/g, respectively.

2.2.6. Lipid extraction and analysis of feces

Collected feces were let dry overnight in fume hood and weighted as about 100 mg prior to more dry for 1 hr at 60℃ in oven. Each stools were extracted in 500 μl of folch solution [chloroform:methanol (2:1)] incubating at room temperature (RT) overnight. After incubation, each sample was spin down to pellet fecal debris at 12000 g RT. The supernatant was transferred and 500 μl 0.9% NaCl was added. It was shaken vigorously for 30 min and centrifuged at 12000 g RT to collect the organic (lower) phase. An aliquot of the organic phase was dried in fume hood overnight and dried lipid was dissolved in 25% Triton X-100 in ethanol. The content of fatty acids in feces was measured using HR series NEFA-HR(2) kit (Wako Chemical, Richmond, VA, USA).

2.2.7. Statistical analysis

All data were expressed as the sample mean ± standard error of the mean (SEM). Statistical mean differences between groups were verified using Student's

t-test. Probability values of $p < 0.05$ and 0.01 were used as criterion for statistical significance.

2.3. Result

2.3.1. SFN suppresses HFD-induced increases in body weight in both Nrf2-WT and Nrf2-KO mice

It has been reported that SFN has an anti-obesity effect (1, 2, 4). To investigate whether Nrf2 is involved in the anti-obesity effect of SFN, both Nrf2-WT and Nrf2-KO mice were fed HFD for 15 weeks with or without SFN 50 mg/kg. Both HFD-fed WT and KO mice were obese up to 50.05% and 45.24%, respectively, compared to each control mice fed CD. I found that SFN significantly suppressed HFD-induced increase of body weight regardless of presence of Nrf2 (Figure 1A and B). SFN significantly reduced the body weight gain in both Nrf2-WT and Nrf2-KO (Figure 1C). I utilized Nrf2-WT and Nrf2-KO mice strains, which were verified before all experiments. The SV40 nuclear localization signal (NLS)-β-galactosidase-*lacZ* recombinant genes were inserted at the coding sequence of exon 5 in *nrf2* in Nrf2 KO mice. Genomic DNA were extracted from mouse tails and amplified for genotyping PCR. I identified this insertion by decreased size of PCR band from 734 bp (WT) to 400 bp (KO) (Figure 1D).

2.3.2. SFN improves HFD-induced impaired insulin sensitivity in both Nrf2-WT and Nrf2-KO mice

Once feeding HFD continuously, obesity gets worse elevating accumulation of ectopic lipid in non-adipose tissues which consequently cause insulin resistance (22, 23). Since it has been reported that SFN relieved type 1

diabetes in streptozotocin-diabetic rats (24, 25), and SFN modulated glucose tolerance and insulin sensitivity in obese mice (26), I examined Nrf2 is involved in the SFN-mediated modulation. On 10 weeks after feeding of HFD with or without SFN (50 mg/kg), Nrf2 WT and KO mice were orally administrated 10% glucose 10 ml/kg for oral-GTT (OGTT) and blood glucose level was measured in regular intervals (Figure 2A and B, respectively). After 2 weeks, i.p. of insulin 1 U/kg was performed in each mice for ITT and blood glucose level was measured in the same intervals as OGTT (Figure 3A and B). In both tests, as shown in graph curve of every interval and area under curve (AUC), which explains the total concentration of blood glucose level over time, HFD-fed mice significantly impaired glucose tolerance compared to CD-fed mice in both Nrf2 WT and KO mice (Figure 2AB and Figure 3AB). Blood glucose level of HFD and SFN-fed mice was recovered more effectively compared to HFD-fed mice (Figure 2AB and Figure 3AB). Consequently, SFN ameliorated insulin resistance in both Nrf2 WT and KO mice, at least in certain interval time.

2.3.3. SFN improves HFD-induced increase in fasting glucose and fasting insulin resulting in improvement of HOMA-IR index in both Nrf2-WT and Nrf2-KO mice

To verify the beneficial effect of SFN on insulin resistance, I analyzed the related parameters such as fasting glucose and fasting insulin in blood plasma. HFD-fed mice showed the increased level of fasting glucose and fasting insulin compared to CD-fed mice. But HFD and SFN (50 mg/kg)-fed mice showed lower

level of fasting glucose and fasting insulin compared to HFD-fed mice in both Nrf2-WT and Nrf2-KO mice (Figure 4A and B). HOMA-IR value also showed that the value of HFD-fed mice was significantly increased than the value of CD-fed mice. The value of HFD and SFN (50 mg/kg)-fed mice was significantly decreased compared to that of HFD-fed mice in both Nrf2-WT and Nrf2-KO mice (Figure 4C). Overall, SFN ameliorated insulin resistance and Nrf2 was not involved in the SFN-mediated modulation.

2.3.4. The anti-obesity effect of SFN is not attributed to decrease of food intake or increase of free fatty acid (FFA) excretion

I examined whether SFN reduced the food intake or increased the lipid excretion in feces. Even though there were no differences of food intake between CD-fed group and HFD-fed group in both WT and KO mice, SFN rather increased the food intake of HFD-fed group in both WT and KO mice (Figure 5A). On the other hand, HFD decreased the FFA excretion to feces compared to CD in both WT and KO mice (Figure 5B). SFN and HFD-fed group showed similar excretion of FFA compared to HFD-fed group in both WT and KO mice (Figure 5B). Consequently, SFN increased food intake but did not influence on FFA excretion in both WT and KO mice. These observations suggest that the anti-obesity effect of SFN was not attributed by the food intake or lipid excretion in both Nrf2 WT and KO mice.

2.4. Discussion

Nrf2 has received attention as an important target protein in the mechanism of SFN's activity for a long time. Since conflicting evidences on the role of Nrf2 in lipid metabolism including obesity have been reported, I investigated the role of Nrf2 in the anti-obesity effect of SFN. I found that SFN suppressed obesity and improved insulin resistance both in Nrf2-WT and Nrf2-KO mice. Consequently, the beneficial effects of SFN on obesity and insulin resistance were independent of Nrf2 (Figure 6).

Chorley *et al*. discovered that SFN mainly modulated Nrf2 and Nrf2-targeted genes, however, some gene groups which still bind to SFN regardless of Nrf2 are detected in ChIP-Seq analysis. Regulation of cell cycle, cholesterol biosynthesis, liver X receptor/retinoid X receptor (LXR/RXR) activation, aryl hydrocarbon receptor signaling, and cholesterol metabolism regulated by hormone receptor were included in those gene groups. Among them, cholesterol biosynthesis related 10 genes were outstandingly responsive to SFN (3). Therefore, they might be the potential molecular targets of SFN to modulate obesity and insulin resistance. Additionally, most previous *in vitro* and *in vivo* studies about the effect of SFN on obesity, have demonstrated that lipid metabolism related pathways including AMP-activated protein kinase (AMPK)-mediated lipogenesis are meaningful signaling pathway for anti-obesity effect of SFN (1, 4). Moreover, as I mentioned in chapter 1, because epigenetics has gradually taken center stage in epidemic obesity (27), it is assumed that epigenetic regulatory pathway is also possible mechanism of SFN's action on obesity in terms that SFN is a dietary histone deacetylase (HDAC) inhibitor (28).

In conclusion, I demonstrate that SFN relieved obesity and insulin resistance even without Nrf2. These results implicate that the action of SFN is not always accompanied by the activation of Nrf2 to show its beneficial effects, at least in obesity and insulin resistance. It is further required to elucidate the concrete mechanisms involved in the regulatory effects of SFN on obesity.

2.5. References

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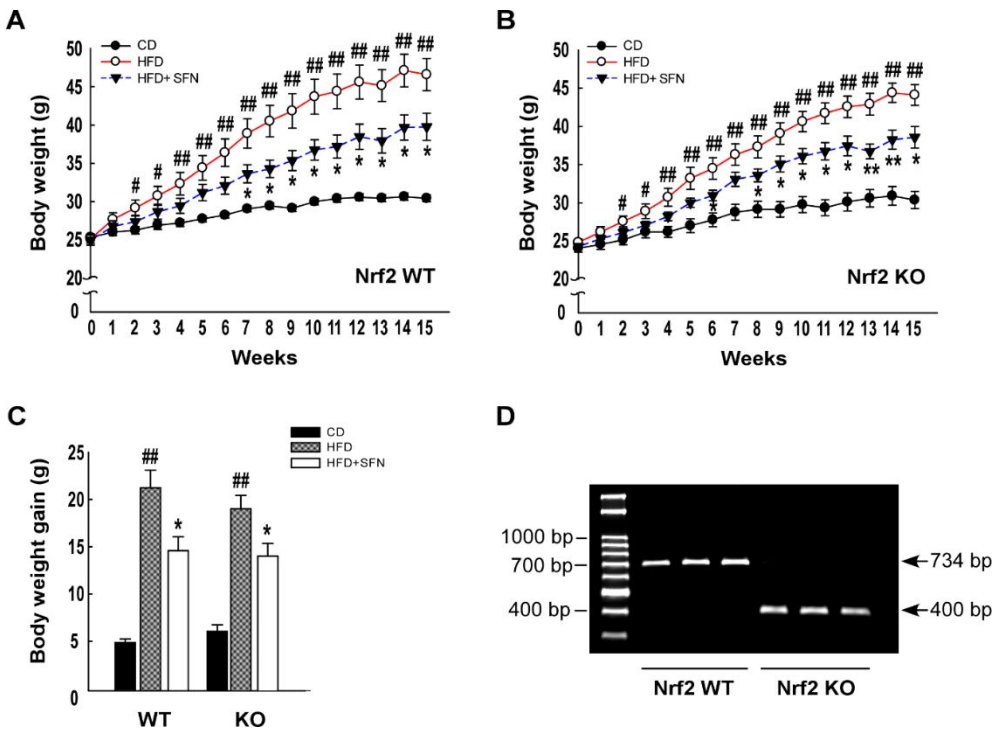

Figure 1. SFN suppresses HFD-induced increases in body weight gain in both Nrf2-WT and Nrf2-KO mice. SFN (50 mg/kg) administrated group reduces body weight gain in HFD-fed Nrf2-WT mice (n= 20) (A). Lowering effect of SFN on body weight is still significant in HFD-fed Nrf2-KO mice (n=19) (B). "Black circles and straight lines = CD + vehicle; white circles and straight lines = HFD + vehicle; black triangles and dashed lines = HFD + SFN (50 mg/kg)." SFN also decreases HFD-induced body weight gain in both Nrf2-WT and Nrf2-KO mice (C). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN (50 mg/kg)." ## $P < 0.01$ vs. CD group mice, * $P < 0.05$ vs. HFD group mice. The genotyped PCR band size of Nrf2-KO mice (400 bp) (n=3) is smaller than one of Nrf2-WT mice (734 bp) (n=3). This is the representative band of amplified genomic DNA (D).

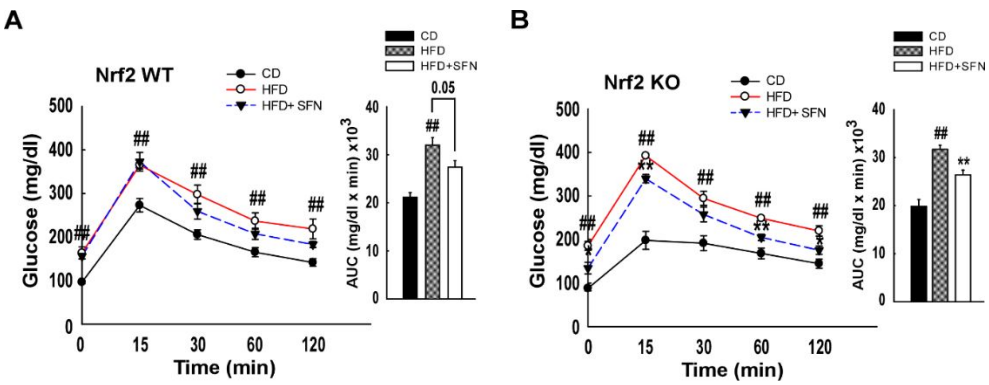

Figure 2. SFN improves glucose intolerance in both Nrf2-WT and Nrf2-KO mice. SFN (50 mg/kg) treated group has lowering effect on blood glucose level compared to the level of HFD group in Nrf2-WT (n=20) (p= 0.05 in AUC) (A). SFN administrated group significantly decrease AUC of OGTT test compared to HFD group in Nrf2- KO mice (n=19), which means SFN recovers HFD-induced glucose intolerance (B). "Black circles and straight lines = CD + vehicle; white circles and straight lines = HFD + vehicle; black triangles and dashed lines = HFD + SFN (50 mg/kg); Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN (50 mg/kg)." Data represents the mean ± SEM. ## P<0.01 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

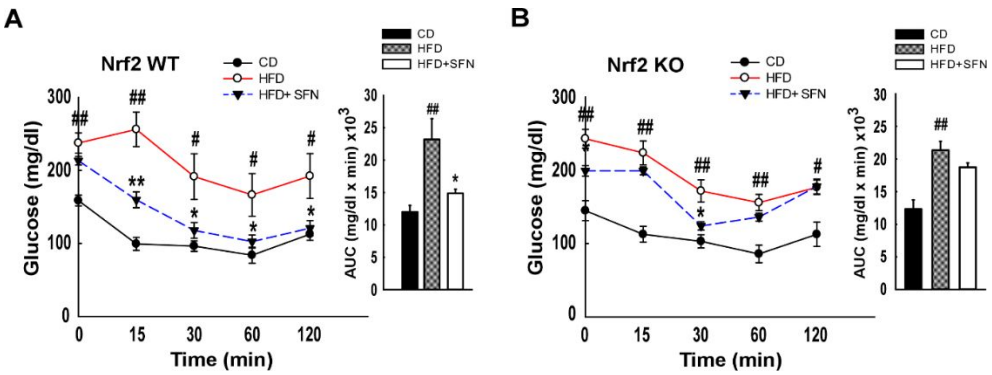

Figure 3. SFN also improves insulin sensitivity in both Nrf2-WT and Nrf2-KO mice. SFN has a beneficial effect on insulin action in both Nrf2-WT (n=20) (A) SFN significantly decrease the glucose level at 30 min in Nrf2-KO mice (n=19) (B). "Black circles and straight lines = CD + vehicle; white circles and straight lines = HFD + vehicle; black triangles and dashed lines = HFD + SFN (50 mg/kg); Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN (50 mg/kg)." Data represents the mean ± SEM. ## P<0.01, # P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

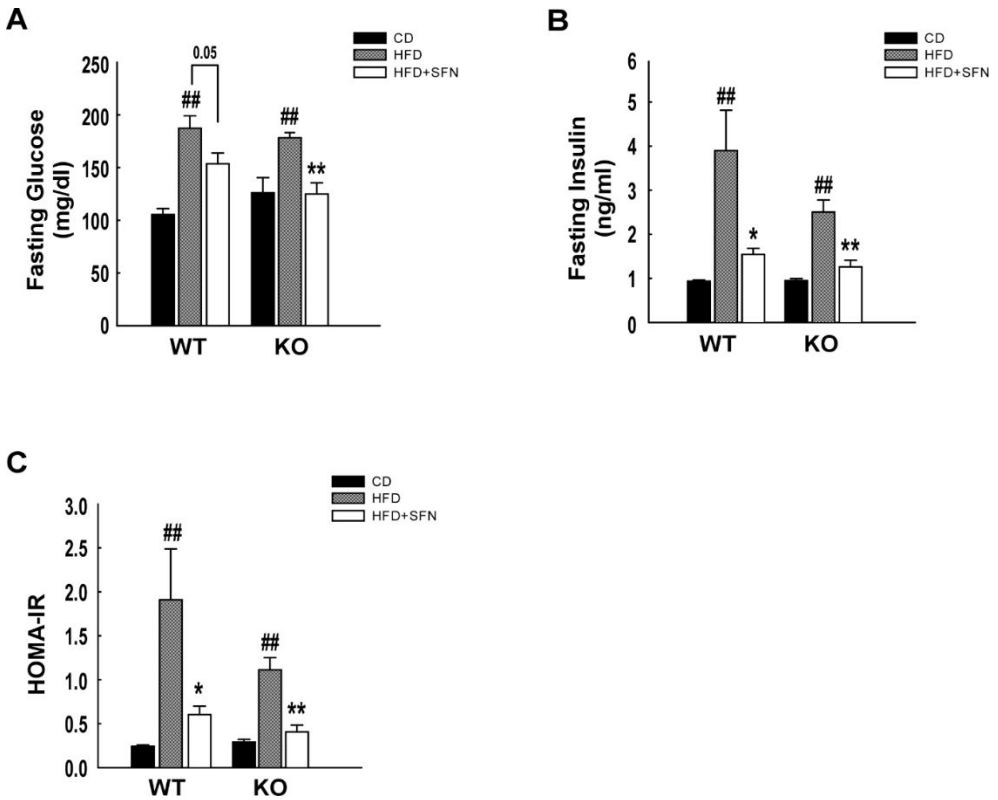

Figure 4. SFN ameliorates HOMA-IR index reducing fasting glucose and insulin level in both Nrf2-WT and Nrf2-KO mice. SFN (50 mg/kg) treatment reduces fasting glucose in plasma of HFD-fed mice (A). Plasma insulin level in SFN administrated group was significantly decreased compared to the level of HFD group (B). HOMA-IR value indicates that HFD induces insulin resistance and SFN improves insulin sensitivity in Nrf2-WT (n=20) and Nrf2-KO-mice (n=19) (C) "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN (50 mg/kg)." Data represents the mean ± SEM. ## $P<0.01$, # $P<0.05$ vs. CD group mice, ** $P<0.01$, * $P<0.05$ vs. HFD group mice.

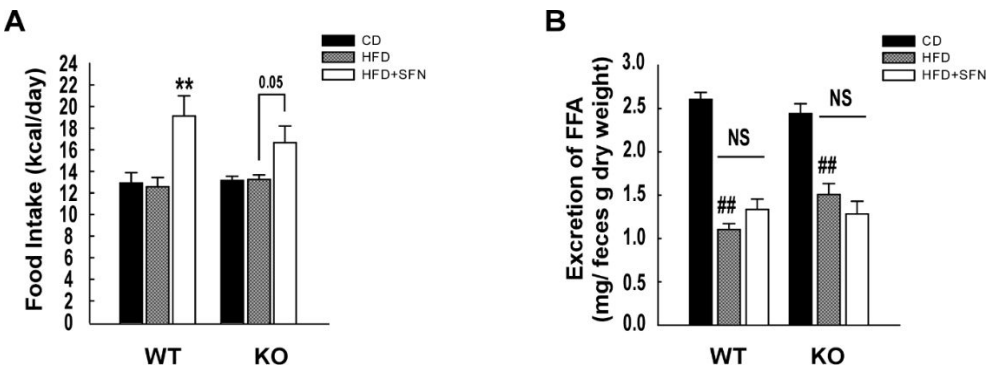

Figure 5. SFN did not either decrease food intake or increase excretion of free fatty acid in feces. SFN (50 mg/kg) treatment rather increases food intake (kcal/day) compared to only HFD feeding (A). There are no significances in excretion of FFA in stool (mg/feces g dry weight) (B). These indicates that reduction of body weight by SFN is not due to decrease of food intake or increase of excretion of FFA in stool. "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN (50 mg/kg)." Data represents the mean ± SEM. ## P<0.01 vs. CD group mice, **P<0.01 vs. HFD group mice.

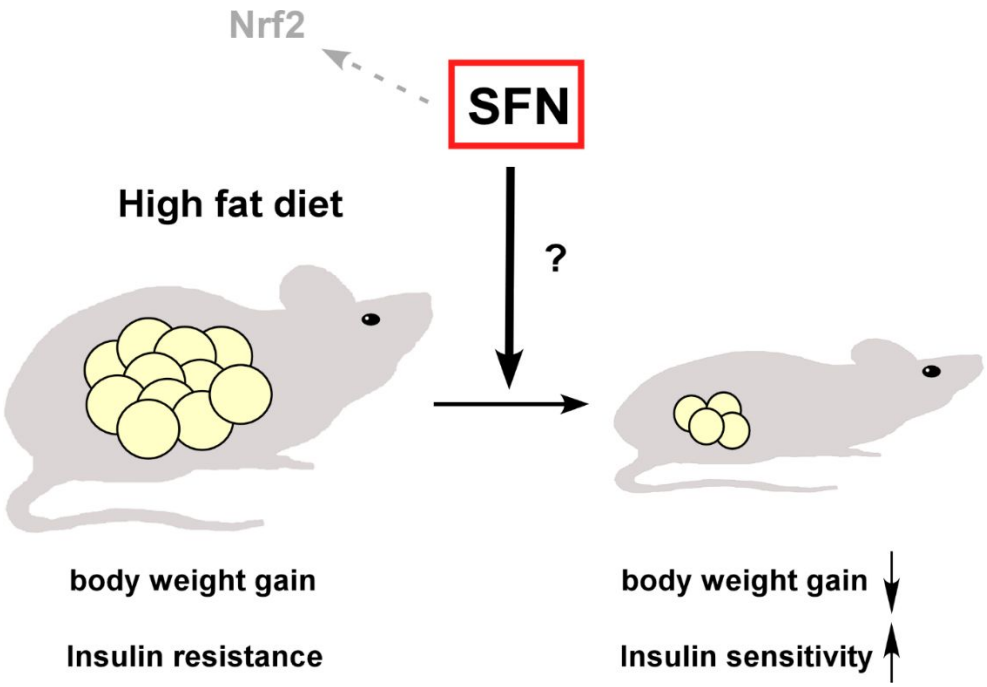

Figure 6. SFN suppresses HFD-induced obesity and improves insulin sensitivity independent of Nrf2 system. Summary. The beneficial action of SFN in HFD fed Nrf2-WT mice is remained even in HFD fed Nrf2-KO mice. This means that SFN attenuates HFD-induced obesity and insulin resistance in HFD-fed mice independent of Nrf2.

Chapter 3

Sulforaphane enhances the lipase expression in white adipose tissue and PGC1α-mediated mitochondrial mechanisms in the liver, muscle, and brown adipose tissue

Abstract

Circulating fatty acids from plasma (released by lipolysis in adipose tissue) as well as lipids stored in each tissues are available to other oxidation-related tissues such as liver, skeletal muscle, brown adipose tissue as a fuel for ATP production. The metabolism of fatty acids including the fatty acid uptake and transport, lipogenesis for storage, fatty acid activation and β-oxidation is a complicate process regulated by multiple regulatory proteins in various tissues. Especially, PGC1α is a master regulator in fatty acid metabolism. PGC1α activates the mitochondrial mechanisms including mitochondrial biogenesis and oxidation binding with various transcription factors such as NRF1/2, PPAR families. Since the abnormality of lipid homeostasis in the body induces dyslipidemia, obesity, and obesity-related metabolic diseases such as type 2 diabetes, it is important to know the strategies for maintaining the lipid homeostasis.

Although there are some studies about anti-obesity effect of SFN, they are limited in terms that most of them have focused on the effect of SFN in adipose tissue. Therefore, for comprehensive understanding of anti-obesity effect of SFN, it is required to examine the systemic effect of SFN in various peripheral tissues.

Here, I investigated the effect of sulforaphane on lipid metabolism in various peripheral tissues such as white adipose tissue, liver, skeletal muscle, and brown adipose tissues to elucidate the underlying mechanisms of its anti-obesity effect. I found that SFN increased the expression of lipases including ATGL and HSL resulting in increase of circulating fatty acids. Simultaneously, I found that SFN also promoted the expression of several proteins involved in PGC1α-mediated mitochondrial biogenesis in the liver and mitochondrial oxidation in the skeletal

muscle, and brown adipose tissues. Overall, these observations demonstrate that SFN contributes the lipid homeostasis in peripheral tissues resulting in alleviative effect on obesity.

Key words: *Sulforaphane (SFN), Fatty acid metabolism. PPARγ coactivator 1- α (PGC1α), lipolysis, mitochondrial biogenesis, mitochondrial β-oxidation*

3.1. Introduction

Fatty acid metabolism involves most tissues, but adipose tissue, skeletal muscle and liver are quantitatively more important than others (1). Each of these tissues has a store of triacylglycerol that can be hydrolyzed (mobilized) to release fatty acids. In adipose tissue, these fatty acids may be released into the circulation for delivery to other tissues, whereas in the liver, muscle, and brown adipose tissue, they are a substrate for oxidation to produce ATP or heat (2-4).

Fatty acid metabolism is tightly regulated by various proteins. Cluster of differentiation 36 (CD36, also known as fatty acid translocase), fatty acid binding proteins (FABPs), fatty acid transport proteins (FATPs) play roles in lipid uptake. Acyl-CoA synthetase long-chain family member 1 (ACSL1) involves in fatty acid activation which change in the form of long-chain acyl-CoA as a key intermediate formed in fatty acid metabolism (5). Fatty acid synthase (FAS) mediates fatty acid synthesis for storage (lipogenesis) (6). Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) regulate break-down of lipid for use (lipolysis) (7, 8). Peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding protein α (C/EBPα) modulate the differentiation of adipocytes (adipogenesis) (9). PPARγ coactivator 1- α (PGC1α) is a transcriptional coactivator found in most cells including skeletal muscle, which regulates the genes involved in fatty acid oxidation (10, 11). PGC1α tightly regulates mitochondrial biogenesis by interacting with various nuclear receptors such as nuclear respiratory factors 1/2 (NRF1/2) or PPAR families including PPARα and PPARβ/δ, promoting the expression of several enzymes related to generating ATP (10, 12). For examples,

carnitine palmitoyltransferase I (CPT-1) mediates fatty acids transportation across the outer mitochondrial membrane to promote fatty acid oxidation processes (13). Apart from PGC1α-mediated fatty acid oxidation, uncoupling protein (UCP) families including UCP1, 2, and 3 play a role mainly in uncoupled fatty acid oxidation which is the process using fatty acid for heat production (14).

For the comprehensive understanding of its anti-obesity mechanism, I investigated the role of sulforaphane (SFN) in the white adipose tissue (WAT), brown adipose tissue, muscle, and liver of high fat diet (HFD)-fed mice. I found that SFN reduces the weight and size of adipocytes and enhances the expression of ATGL and HSL in WAT of HFD-fed mice. In the liver, SFN reduced fat accumulation, especially triglycerides. SFN increased the level of PGC1α, NRF1, and PPARα and enhanced mitochondrial biogenesis in the liver of HFD-fed mice. SFN increased the level of PGC1α in skeletal muscle as well. ACSL1, mitochondrial uncoupling protein 3 (UCP3), CPT-1, and PPARβ/δ were also increased by SFN in the skeletal muscle of HFD-fed mice. In the brown adipose tissue of HFD-fed mice, SFN increased the levels of PGC1α, NRF1, PPARβ/δ and UCP1. These observations suggest that the anti-obesity effect of SFN might be mediated by enhanced ATGL and HSL-induced lipolysis in WAT and PGC1α-modulated free acid oxidation in the liver, muscle, and brown adipose tissue.

3.2. Materials and Methods

3.2.1. Animals

C57BL/6J male mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed with free access to food and water in the animal facility of the Seoul National University in temperature-, light- , and humidity-controlled rooms with a 12-h light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-110126-8).

3.2.2. Treatment

Age-matched male mice were fed a standard diet (STD) (10 kcal% Fat, Research Diets, New Brunswick, NJ, USA) or chow diet (CD) as a control, or a HFD (60 kcal% Fat, Research Diets). Mice were divided into three or four groups (n=6-9 per group): (1) STD (or CD) + phosphate-buffered saline (PBS) control group, (2) HFD + PBS group, and (3-4) HFD + SFN (10 or 50 mg/kg) group. SFN (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in PBS and orally administrated every day for 8 weeks or 15 weeks.

3.2.3. Pearson's correlation study

Pearson's correlation study was used for evaluation of associations between variables. Statistical analyses were determined with the level of

significance at $p < 0.05$. Data were analyzed using GraphPad Prism (Version 4.03, GraphPad Software, San Diego, CA, USA).

3.2.4. Tissue histology

Epididymal WAT (eWAT) and liver were harvested and fixed with 4% formaldehyde in PBS solution. Each tissue was embedded in paraffin for sectioning and hematoxylin and eosin (H&E) staining, and examined at 200 X magnification. The average size and distribution of adipocytes in eWAT were measured using Image J (US National Institutes of Health, Bethesda, MA, USA).

3.2.5. Serum biochemistry

Serum non-esterified fatty acids (NEFA) were determined with a quantification assay kit (Wako Chemical, Richmond, VA, USA). Triglycerides in the serum were also examined with a serum triglyceride determination kit (Sigma, Saint Louis, MO, USA). Using spectrophotometer, the absorbance was measured at 550 nm for NEFA and 540 nm for triglyceride. Total cholesterol and high-density lipoprotein (HDL) cholesterol were determined by using HDL-cholesterol kit (Asanpharm, Seoul, Korea). Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald Formula of [Total cholesterol]-[HDL-cholesterol] - [Triglyceride/5] with substitution of each concentration value (mg/dL) (15).

3.2.6. Lipid extraction and analysis of liver

Lipid accumulated in the liver was quantified by modified the "Folch" Procedure (16). Briefly, livers were weighted as about 100-150 mg and homogenized in 3 mM $CaCl_2$. The homogenate (2.5 ml) was extracted with 10 ml of folch solution [chloroform:methanol (2:1)]. An aliquot of the organic phase was collected and dried in fume hood overnight. Dried lipid was dissolved in 25% Triton X-100 in ethanol. Hepatic triglyceride and total cholesterol were determined as described in Serum Biochemistry.

3.2.7. Western blot assay

Tissues were harvested and homogenized in radio immunoprecipitation assay buffer (RIPA buffer) (Cell signaling, Danvers, MA, USA) with one tablet of protease inhibitor cocktail (Roche, Penzberg, Germany). After centrifugation (14000 rpm, 10 min), the supernatants of homogenates were collected and analyzed to determine the concentration of proteins using protein assay kit (Bio-Rad, Hercules, CA, USA). The tissue lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE healthcare, Piscataway, NJ, USA). The membrane was blocked with 5% skim milk and incubated with specific primary antibodies. Antibodies against ACSL1 (1:1000), NRF1 (1:1000), ATGL (1:1000), and HSL (1:1000) were obtained from Cell Signaling. Antibodies against PGC1α (1:1000) and UCP3 (1:1000) were purchased from Abcam (Cambridge, MA, USA). Antibodies against CPT1M (1:1000), PPARα (1:1000), PPARβ/δ (1:1000) and

UCP1 (1:1000) were obtained from Santa Cruz. Antibody against GAPDH was obtained from Sigma. After incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma, 1:5000) for 1 hour at room temperature, the protein bands were visualized using a chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA). Band intensities were quantified with Image J.

3.2.8. Quantitative real time-polymerase chain reaction (qRT-PCR)

Tissues were homogenized in RNA iso plus (Takara, Dalian, China) and total RNA was isolated using the RNA Mini kit (Ambion, Foster City, CA, USA). cDNA was synthesized by PrimeScript RTase (Takara). Quantitative RT-PCR reaction was performed using CFX96 real-time PCR detection system (Bio-Rad). cDNA was amplified in the presence of SYBR Green (Bio-Rad). Relative expression levels of mRNA were calculated according to ddCt method (2^{-ddCt}) (17). The primer sequences were described in table 1.

Table 1. The primer sequences for qRT-PCR

Gene	F/R	Sequence
Cd36	Forward	5′-ATGGGCTGTGATCGGAACTG-3′
	Reverse	5′-GTCTTCCCAATAAGCATGTCTCC-3′
Fasn	Forward	5'-TTGCCCGAGTCAGAGAACC-3'
	Reverse	5'-CGTCCACAATAGCTTCATAGC-3'
Cebpα	Forward	5'-CGCAAGAGCCGAGATAAAGC-3'
	Reverse	5'-CACGGCTCAGCTGTTCCA-3'
Hsl	Forward	5′-GCCGGTGACGCTGAAAGTGGT-3′

	Reverse	5′-CGCGCAGATGGGAGCAAGAGGT-3′
Atgl	Forward	5′-GAGCCCCGGGGTGGAACAAGAT-3′
	Reverse	5′-AAAAGGTGGTGGGCAGGAGTAAGG-3′
Nqo1	Forward	5′-TTCTCTGGCCGATTCAGAGT-3′
	Reverse	5′-GGCTGCTTGGAGCAAAATAG-3′
Ho-1	Forward	5′-CAGGTGATGCTGACAGAGGA-3′
	Reverse	5′-GAGAGTGAGGACCCACTGGA-3′
Gclc	Forward	5'-ATGTGGACACCCGATGCAGTATT-3'
	Reverse	5'-TGTCTTGCTTGTAGTCAGGATGGTTT-3'
Gclm	Forward	5'-GCCACCAGATTTGACTGCCTTT-3'
	Reverse	5'-CAGGGATGCTTTCTTGAAGAGCTT-3'
Nrf2	Forward	5'-TCACACGAGATGAGCTTAGGGCAA-3'
	Reverse	5'-TACAGTTCTGGGCGGCGACTTTAT-3'
Fabp4	Forward	5'-CATGGCCAAGCCCAACAT-3'
	Reverse	5'-CGCCCAGTTTGAAGGAAATC-3'
Scd-1	Forward	5′-CCAGAATGACGTGTACGAATGG-3′
	Reverse	5′-GCGTGTGTTTCTGAGAACTTGTG-3′
Srebp1c	Forward	5′-CTGGCACTAAGTGCCCTCAAC-3′
	Reverse	5′-GCCACATAGATCTCTGCCAGTGT-3′
Dgat1	Forward	5′-TTTGTTGTGGCTGCATTTCAG-3′
	Reverse	5′-TGATTGTGGCCAGGTTAACCA-3′
ApoB	Forward	5′-CAGGCTGATGCTGTTTTGAA-3′
	Reverse	5′-CTGAGGGATTTGGGATCAGA-3′
Mtp	Forward	5'-TGAGCGGCTATACAAGCTCAC-3'
	Reverse	5'-CTGGAAGATGCTCTTCTCGC-3'
Pparγ	Forward	5'-CGCTGATGCACTGCCTATGA-3'

	Reverse	5'-AGAGGTCCACAGAGCTGATTCC-3'
Fatp5	Forward	5'-ACTCTGTACGGAGCTCTGGG-3'
	Reverse	5'-GAGCTGTGGCCAAGGTAGAA-3'
Fapb1	Forward	5'-CATCCAGAAAGGGAAGGACA-3'
	Reverse	5'-TTTTCCCCAGTCATGGTCTC-3'
β-actin	Forward	5'-TGTCCACCTTCCAGCAGATGT-3'
	Reverse	5'-AGCTCAGTAACAGTCCGCCTAGA-3'

3.2.9. DNA isolation and mitochondrial DNA (mtDNA) content

Tissues were homogenized in lysis buffer [100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, and 100 μg/ml Proteinase K] and incubated overnight at 55^{o}C in humidified condition. After isopropanol was added, the homogenates were shaken until DNA precipitates became visible. DNA pellets were washed with 70% Ethanol twice and dried for 10 minutes at room temperature. Isolated DNA pellets were dissolved in Tris-EDTA buffer. Quantification of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) was performed by quantitative RT-PCR reactions, using specific primers for the mtDNA encoded cytochrome B gene and the nDNA encoded 18S ribosomal RNA (rRNA) gene as described in Table 2. mtDNA copy number was determined as mtDNA/nDNA ratio, in which each value was calculated by using the formula: $2 \times 2 (\Delta C_T)$ (18).

Table 2. The primer sequences for nDNA and mtDNA

Gene	F/R	Sequence
Cytochrome B	Forward	5'-CCACTTCATCTTACCATTTATTATCGC-3'

	Reverse	5'-TTTTATCTGCATCTGAGTTTAATCCTGT-3'
18S rRNA	Forward	5'-GTAACCCGTTGAACCCCATT-3'
	Reverse	5'-CCATCCAATCGGTAGTAGCG-3'

3.2.10. Statistical analysis

All data were expressed as the sample mean ± standard error of the mean (SEM). Statistical mean differences between groups were verified using Student's t-test. Probability values of $p < 0.05$ and 0.01 were used as criterion for statistical significance.

3.3. Result

3.3.1. SFN decreases the weight of eWAT and the size of adipocytes

To elucidate the effects of SFN on eWAT, the weight of eWAT and the size of adipocytes were investigated in mice fed STD or HFD with or without SFN (10 and 50 mg/kg) for 8 weeks. As a representative adipose tissue, I compared the weight of eWAT among groups. Pearson's correlation study was carried out to analyze the correlation between the body weight and the weight of eWAT altered by SFN. I found that SFN decreased the weight of eWAT (Figure 1A) and the reduction of body weight by SFN was strongly correlated with the decrease of eWAT weight by SFN (Figure 1B). On the other hand, H&E staining of eWAT showed that the adipocytes of eWAT in HFD-fed mice were larger than those in STD-fed mice. SFN treatment (10 or 50 mg/kg) significantly reduced the average size of adipocytes in the eWAT of HFD-fed mice (Figure 1C and D). Additionally, the number of adipocytes, which have cell surface area over 6000 mm^2, was significantly less in eWAT of HFD + SFN (10 or 50 mg/kg) group, compared to HFD group (Figure 1E).

3.3.2. SFN increases the expression of ATGL and HSL in eWAT

To identify the mechanisms associated with SFN-mediated anti-obesitic changes in eWAT, I analyzed the genes regulating lipid metabolism in eWAT using Western blot analysis and qRT-PCR. I found that SFN treatment significantly increased the protein levels of ATGL and HSL, lipases involved in lipolysis in WAT (Figure 2A and B). Moreover, SFN treatment also significantly increased the mRNA

expression of Atgl and Hsl, consistent with the increased protein level (Figure 2C and D). On the other hand, administration of SFN did not show significant change in other group genes including peroxisome proliferator-activated receptor γ (Pparγ), and CCAAT-enhancer-binding proteins α (C/epbpα) related to adipogenesis (differentiation of preadipocyte to adipocyte) and fatty acid synthase (Fasn or Fas) related lipogenesis (synthesis of lipid), cluster of differentiation 36 (Cd36), and fatty acid binding protein 4 (Fabp4) related to fatty acid transport (lipid uptake into adipocytes) in WAT (Figure 2E). These results indicate that lipase-mediated lipolysis may contribute to the reduction of the weight of eWAT rather than other mechanisms in adipose tissue such as lipogenesis, adipogenesis.

3.3.3. SFN changes lipid profile in serum

Since SFN increased the expression of lipase in WAT in Figure 2, main action of SFN might not be limited just in WAT. Free fatty acid would be released to other organs such as liver, muscle, and brown adipose tissue, which are involved in lipid metabolism. Thus, I investigated how lipid factors in serum including NEFA level were changed by SFN. Consistently with increase of mRNA expression of lipase, NEFA, and glycerol level in serum of HFD+SFN 50 mg/kg group were significantly increased compared to HFD only fed group (Figure 3A and B). Additionally, administration of SFN also increased triacylglycerol (TG) level (Figure 3C). The level of total cholesterol, HDL and LDL cholesterol were significantly increased by feeding HFD but there are no significant difference between HFD only fed group and HFD+SFN 50 mg/kg group (Figure 3D-F).

3.3.4. SFN inhibits the fat accumulation in liver.

From lipid profile in serum, SFN treatment increased the level of TG as well as NEFA. Since TG or cholesterol is synthesized from fatty acids in liver, I investigated the effect of SFN on lipid metabolism in liver. H&E data showed that HFD significantly increased the lipid droplet in liver. And HFD + SFN (10 or 50 mg/kg) mice had less lipid droplet in liver compared to HFD-fed mice. This result indicated that administration of SFN inhibited the fat accumulation in liver (Figure 4A). Additionally, I quantified the level of TG and cholesterol in liver. Although HFD did not increase the level of hepatic TG (mg/g) compared to STD, SFN (50 mg/kg) treatment significantly reduced the level of hepatic TG than only HFD feeding (Figure 4B). On the other hand, there was no significant change in the level of hepatic cholesterol among STD, HFD, and HFD+SFN group (Figure 4C). These observations suggested that fatty acid released from adipose tissue to serum by SFN did not cause fat accumulation in liver.

3.3.5. SFN increases the PGC1α-mediated mitochondrial biogenesis in liver.

PGC1α a master regulator protein against mitochondrial biogenesis and mitochondrial oxidation, plays an important role in fatty acid metabolism in liver interacting with its coactivators such as NRF1, and PPARα, consequently resulting in induction of mitochondrial biogenesis or fatty acid oxidation (19). Thus, I examined the expression of not only PGC1α protein but also its coactivators,

NRF1, and PPAR protein to elucidate the mechanism of reducing effect of SFN on lipid accumulation in liver. HFD significantly reduced the expression of PGC1α and SFN (10 or 50 mg/kg) treatment significantly recovered the expression of PGC1α Figure 5A and B). While HFD did not significantly change the expression of NRF1 compared to STD, SFN treatment (50 mg/kg) remarkably increased the NRF1 protein expression (Figure 5A and C). SFN (50 mg/kg) administration also up-regulated the protein expression of PPARα reduced by HFD feeding (Figure 5A and D). Moreover, to more investigate whether the increase of mitochondrial biogenesis resulted from the remarkable up-regulated expression of PGC1α and NRF1 by SFN, I quantified mtDNA content. The mtDNA content was determined as relative expression of mitochondrial encoded gene such as cytochrome B normalized by the expression of nuclear encoded gene such as 18s ribosomal RNA. Although there was no significance between HFD fed mice and STD fed mice, HFD+SFN (10 or 50 mg/kg) fed mice significantly increased mtDNA content compared to HFD only fed mice (Figure 5E). These observations indicated that SFN increase the mitochondrial biogenesis up-regulating the expression of PGC1α and its co-activators, followed by fatty acid oxidation in liver.

3.3.6. SFN does not affect gene expressions in other lipid metabolic pathway in liver: lipid uptake, lipid transportation, lipid biosynthesis.

To examine the effect of SFN in other lipid metabolic gene expression in liver, I carried out qPCR. However, I did not found any differences in the

expression of genes such as Pparγ, sterol regulatory element-binding protein 1c (Srebp1c), Fasn, stearoyl-CoA desaturase-1 (Scd-1), and diacylglycerol O-acyltransferase 1 (Dgat-1) associated to biosynthesis of lipid and cholesterol. In addition, SFN (10 or 50 mg/kg) treatment did not change the expression of genes involved in fatty acid transport such as Cd36, fatty acid transport protein 5 (Fatp5), and fatty acid binding protein 1 (Fabp1), genes related to the synthesis of lipoprotein such as apolipoprotein B (ApoB), and microsomal triglyceride transfer protein (Mtp) (Figure 6A-C). Overall, these results suggested that reduction of fat accumulation in liver in HFD+SFN (10 or 50 mg/kg) fed mice observed in Figure 5A is attributed not to decrease of lipid uptake or lipogenesis, increase of secretion of lipoprotein but to increase of the number of mitochondria with oxidation ability.

3.3.7. SFN enhances PGC1α-mediated mitochondrial fatty acid oxidation in skeletal muscle.

Otherwise excess lipid factors in blood stream were appropriately removed, insulin resistance would get worse resulting in increasing the risk of certain metabolic disorders such as type 2 diabetes (20). So in order to elucidate where released free fatty acid (FFA) and TG to go and how they were eliminated, I next focused on muscle where fatty acid oxidation is mainly occurred because it is enrich mitochondria utilizing fatty acid as an energy source. As in liver, PGC1α modulates overall fatty acid oxidation by transcriptional activation of catabolic enzymes related to mitochondrial biogenesis and oxidation in serial action of other proteins such as ACSL1, UCP3, CP1-1M, PPARβ/δ in skeletal muscle (21, 22).

HFD decreased the expression of PGC1α compared to STD, and SFN (50 mg/kg) administration remarkably up-regulated the PGC1α expression (Figure 7A and B). While HFD significantly reduced the expression of ACSL1, a protein activating fatty acid to utilize for energy production, compared to CD, SFN (50 mg/kg) administration significantly increased the ACSL1 expression compared to HFD (Figure 7A and C). SFN (50 mg/kg) treatment also increased the expression of UCP3 slightly decreased by HFD, which is an important protein for catalyzing mitochondrial lipid oxidation (Figure 7A and D). Additionally, HFD feeding lowered the expression of CPT-1M, a protein involved in transportation of fatty acid from outer membrane of mitochondrial to inner membrane of mitochondria for oxidation, and SFN (50 mg/kg) administration recovered it (Figure 7E and F). SFN (50 mg/kg) administration also significantly increased the expression level of PPARβ/δ in HFD fed mice (Figure 7E and G). These results suggested that SFN enhanced PGC1α GC1t SFN fatty acid oxidation by up-regulation of oxidation-related proteins expression.

3.3.8. SFN enhances PGC1α-mediated thermogenesis in brown adipose tissue.

Brown adipose tissue (BAT) as well as skeletal muscle is also oxidative organ for energy expenditure. As in liver and muscle, the expression of PGC1α, NRF1, PPARβ/δ protein was measured in BAT to elucidate the effect of SFN on mitochondrial biogenesis and fatty acid oxidation using western blot. Although HFD slightly influenced the expression of proteins, administration of SFN (50

mg/kg) significantly increased the expression of PGC1α, NRF1, PPARβ/δ (Figure 8A-D). Furthermore, in brown adipose tissue, fatty acids are utilized in thermogenesis which is the process of heat production as an important component of the metabolic rate. Thus, I examined the expression of UCP1 as a representative marker protein of thermogenesis. Despite HFD did not change the UCP1 expression, administration of SFN (50 mg/kg) increased the expression of UCP1 remarkably (Figure 8A and E).

3.4. Discussion

Imbalance between lipid uptake and release, synthesis and disposal can cause metabolic disorders as well as obesity (23). Various regulatory proteins are harmonized to control homeostasis of lipid metabolism depends on metabolic responses in each peripheral tissues. Firstly, adipose tissue is important to store lipid as an energy source. When we eat food, FFAs or TGs are absorbed from intestine. And they are transported in the form of chylomicron to adipose tissue. CD36 and FAPB4 mediated the uptake of fatty acids. Fatty acids are processed for lipogenesis and stored in the form of TG. Whenever our body needs energy, lipase such as ATGL, and HSL triggers TG to break down to FFAs. FFAs are released into blood (lipolysis) and transferred to other peripheral tissues such as liver, muscle, brown adipose tissue as sources of energy production (8).

It has been reported that the mice lacking ATGL disrupted lipolysis and energy metabolism. Increase of ATGL expression promotes lipolysis and is helpful for reinforcing energy metabolism (20). Additionally, Some regulatory proteins involved in adipogenesis, and lipogenesis such as PPARγ, C/EBPα, FAS, are important key factors to keep balance between efflux and influx of lipid (24).

Free fatty acids in blood are used for energy or participated in de novo synthesis of other lipid factors such as TG, and cholesterol in other organ. Secondly, liver is important to adjust TG and cholesterol metabolism (8). Liver takes up FFAs or TG through CD36, which is a membrane receptor. Lipogenesis-related proteins such as PPARr, FAS, SCD1, DGAT1, and SREBP1c regulate de novo lipogenesis converting FFAs to TG or cholesterol. CPT-1a, and PPARα control the FFA oxidation in liver. PGC1α and its co-activator NRF1 mediate

mitochondrial biogenesis enhancing fatty acid oxidative metabolism in liver. In addition, liver secretes TG and cholesterol to blood stream in the form of lipoprotein such as very low-density lipoprotein (VLDL). In this process, lipoprotein synthesis-related proteins such as apoB, MTP mediate and pack TG, and cholesterol in the lipoproteins, which is secreted into the blood (20).

Various lipid factors in blood such as FFA or TG can be used as a fuel source in muscle. Thirdly, muscle is important to burn fatty acids in mitochondria to produce energy as an ATP. Fatty acid oxidation in muscle usually contains a series of three steps (5). In first step, fatty acid is activated and transported into the mitochondria because enzymes involved in fatty acid oxidation are mainly located in mitochondrial matrix. Once fatty acids are taken up into the tissue, ACSL1 catalyzes the activation of fatty acids to long-chain acyl-CoA, which is a pre-step for beta-oxidation (5). CPT-1 triggers transportation of fatty acids across the outer mitochondrial membrane in the form of acyl carnitine followed by next process to produce acetyl-CoA, an entry form for citric acid cycle, so called TCA cycle. Therefore, this step is regarded as a rate-limiting step in whole process of fatty acid oxidation (8). β-oxidation is a second step for fatty acids to be undergone after transportation inside the mitochondria. β-oxidation is the process by which fatty acid are broken down Acyl-CoA molecules in mitochondria followed by eventually enter into the TCA cycle in the form of acetyl-CoA. This step is prerequisite to next step. Electron transport chain is a final step in fatty acid oxidation to produce ATP as an energy source. During this process, UCP3, a downstream protein of PGC1α in mitochondrial inner membrane, exports the remaining fatty acid anions which are not oxidized and remained in mitochondria to the cytosol for recycling

them. This is allowed to maintain this fatty acid oxidation cycle continuously by reactivating fatty acids into acyl CoA rapidly in the first steps (25). PPARβ/δ, and PGC1α, in nucleus, trigger and boost this fatty acid catabolism promoting transcription of fatty acid oxidative genes (24, 26-28). Moreover, PGC1α also promotes fatty acid catabolism by inncrease of mitochondrial biogenesis in addition to mitochondrial fatty acid oxidation, which is regulated by NRF-1, NRF2 as co-activators in skeletal muscle.

Brown adipose tissue as well as muscle is also one of oxidative organs contributing energy expenditure. Many oxidation-related proteins such as PGC1α, PPARβ/δ, and NRF1 in muscle play similar roles in brown adipose tissue. However, thermogenesis mediated by UCP1 also occurred in brown adipose tissue as other consuming way of lipid source, which is an uncoupled oxidation accompanying a heat production rather than ATP production.

However, some gain-of function experiments suggested that PGC1α KO mice have normal mitochondrial volume in muscle and were rather lean. And these results seems to show that PGC1α is not required in mitochondrial biogenesis *in vivo* (21). Nevertheless, it is clear that obesity, diabetes and several metabolic disorders negatively correlate with the level of PGC1α (29, 30). And despite more research is required in role of PGC1α, the increase of PGC1α expression activates global mitochondrial function and consequently exerts metabolic benefits against obesity and other metabolic diseases (10).

Consequently, SFN induced release of FFAs from adipose tissue to the blood stream by activating lipases such as ATGL, HSL. And administration of SFN induced the increase of TG level as well as FFA level in blood, which increase of

food intake might contribute. And then either FFA or TG was utilized as a fuel of energy metabolism in liver, muscle, and brown adipose tissue. PGC1α, ACSL1, CPT1, PPAR families, and UCP1 or 3 participated and mediated in stage by stage. Especially, the high level of FFA and TG (Figure 4A and C) indicated that SFN might accelerated fatty acid catabolism by promoting provision of lipid source from adipose tissue or perhaps intestine. In summary, SFN enhances fatty acid metabolism starting with PGC1 α in HFD-fed mice resulting in alleviation of obesity in Nrf2 pathway independent manner (Figure 9).

In conclusion, I revealed more concrete mechanism of SFN via PGC1 α in each peripheral tissue such as adipose tissue, liver, and muscle in this chapter. However, the action of PGC1α is modulated by sequential multiple steps including transcription, translation, post-translation such as acetylation, ubiquitination and so on. Therefore, it is still remained how SFN regulated PGC1α resulting in beneficial action in obesity.

3.5. References

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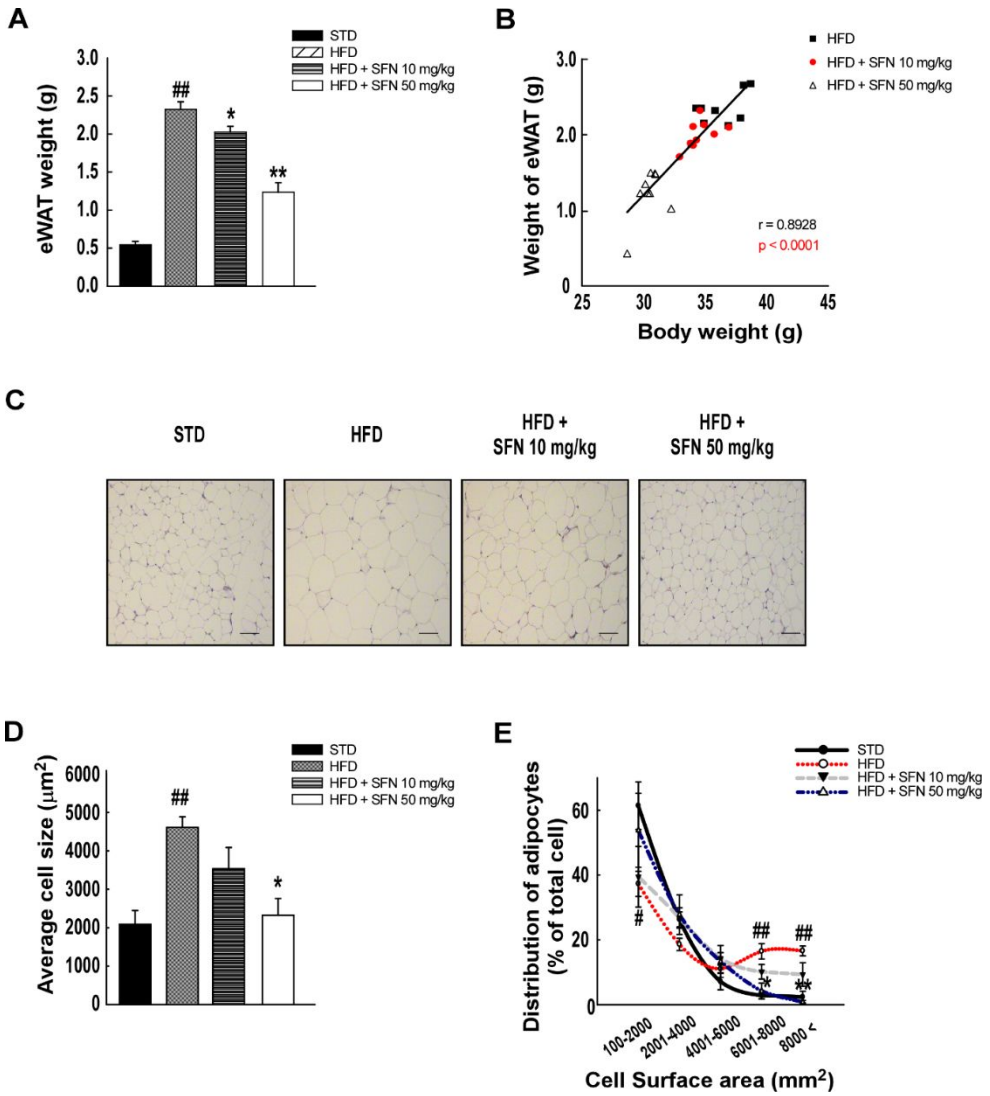


Figure 1. SFN reduces the size and weight of eWAT in HFD-fed mice. SFN reduced the weight of eWAT compared to HFD in a dose dependent manner. This was a modified data retrived from unpublished mastuer's thesis. (Hee Yang. "Anti-obesity effect of sulforaphane through suppressing Ras activity *in vitro* and in *vivo*". Master's thesis, Seoul National University, 2010) (A). The reduction of body weight and decrease of eWAT weight by SFN (10 or 50 mg/kg) had positive correlation (B). "Black square = HFD; red circles = HFD + SFN 10 mg/kg; white

triangles = HFD + SFN 50 mg/kg." H&E stained WAT (X200) histological data showed that size of adipocyte in adipose tissue of SFN group is similar to STD group (C). The average size of adipocytes in epididymal fat was also decreased by SFN (D). WAT of SFN treated group contained more small sized adipocytes (<6000 mm^2) than big ones (> 6000 mm^2) compared to only HFD fed group (E). Data represents the mean ± SEM. "Black straight line = STD + vehicle; Red straight line = HFD + vehicle; Dotted line = HFD + SFN 10 mg/kg; Broken line = HFD + SFN 50 mg/kg."

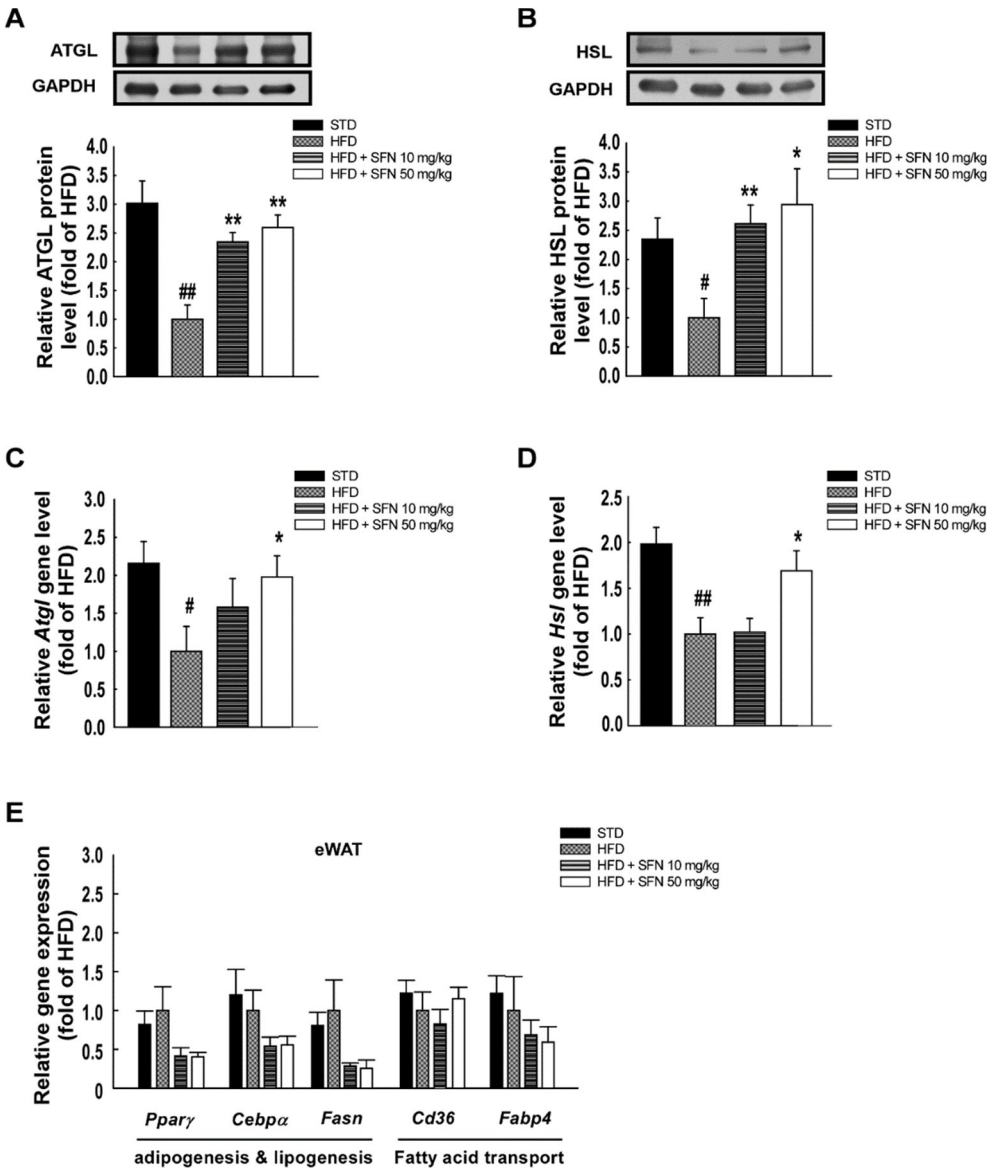


Figure 2. SFN induces the expression of lipase such as ATGL, and HSL involved in lipolysis in epididymal WAT. Relative expression of ATGL (A) and HSL (B) proteins were suppressed by SFN (10 or 50 mg/kg) treatment in a dose dependent manner. SFN also up-regulated relative expression of *Atgl* gene (C) and *Hsl* gene (D) in HFD–fed mice. However, SFN had no significant effect on not only adipogenesis, lipogenesis related genes such as *Pparγ*, *Cebpα*, *Fasn* genes but

also *Cd36*, *Fabp4* genes which were fatty acid transport related genes (E). “Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg.” Data represents the mean ± SEM. $^{\#\#}$ P<0.01 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

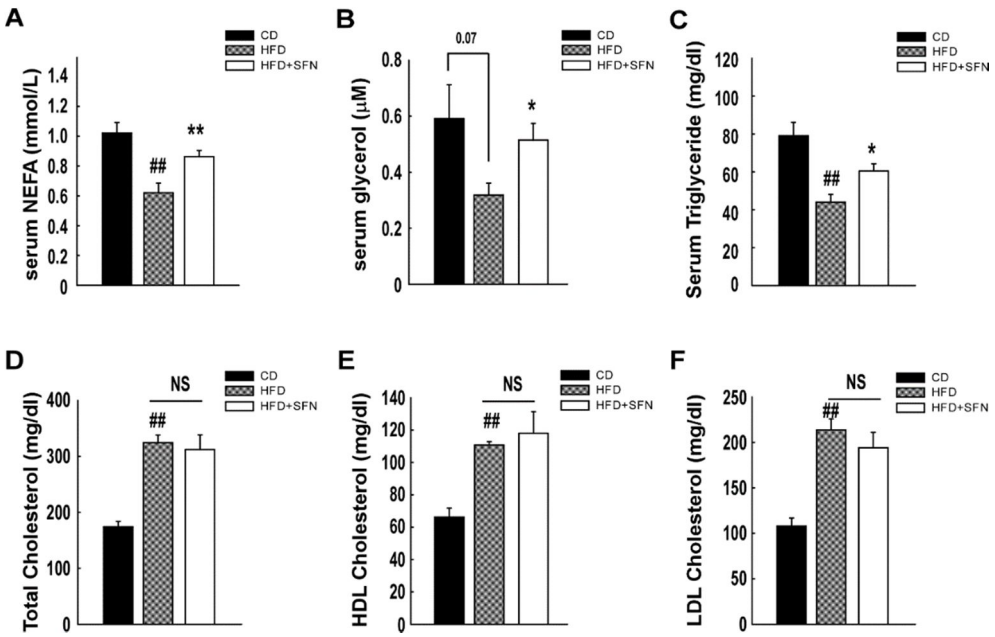


Figure 3. SFN alters the lipid profiles in the blood serum. The level of NEFA (mmol/L) (A) and glycerol (μM) (B) were increased in blood serum by increase of lipolysis. The level of triglyceride (TG) (mg/dl) in blood serum HFD+SFN 50 mg/kg group was also increased compared to the level in blood serum of only HFD fed group (C). On the other hand, there were no significant changes in the level of total cholesterol (D), HDL-C (E), LDL-C (F). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice, NS: no significance.

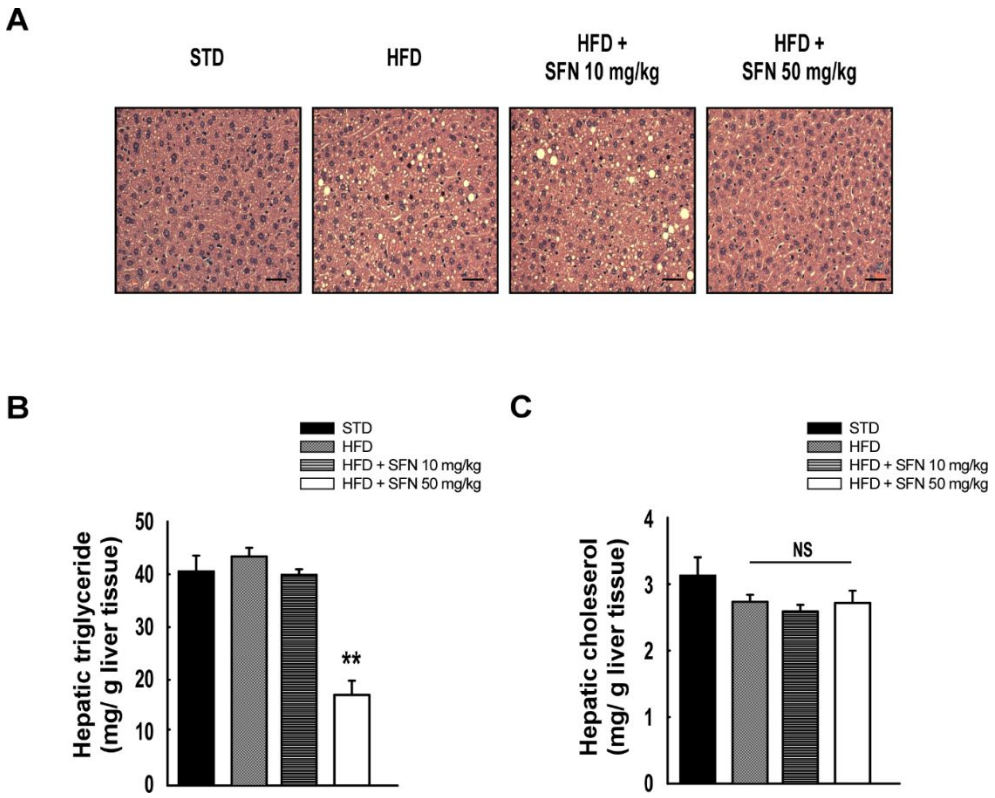


Figure 4. SFN lowers the accumulation of fat in liver. In histological picture, SFN decreased the accumulation of lipid in liver (A). SFN (50 mg/kg) administration remarkably decreased hepatic triglyceride compared to HFD (B). There was no significant difference in hepatic cholesterol among all groups (C). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. **P<0.01, vs. HFD group mice, NS: no significance.

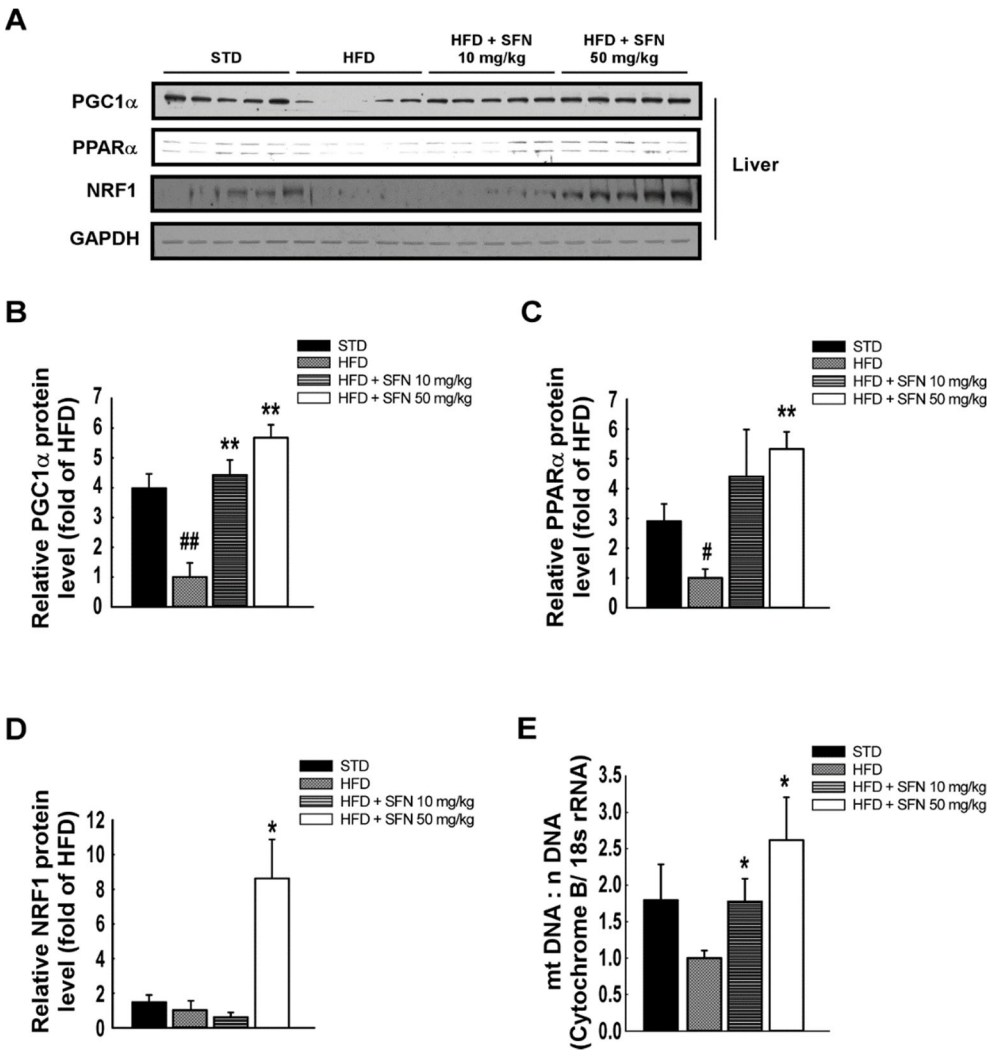
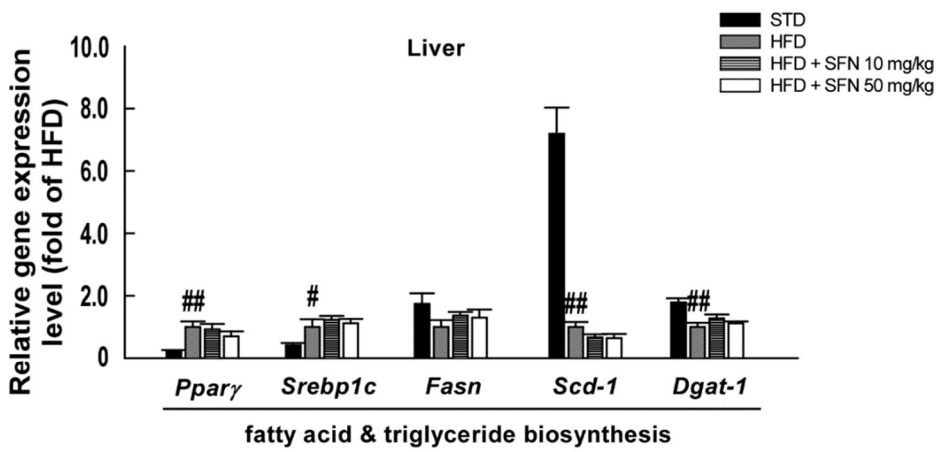


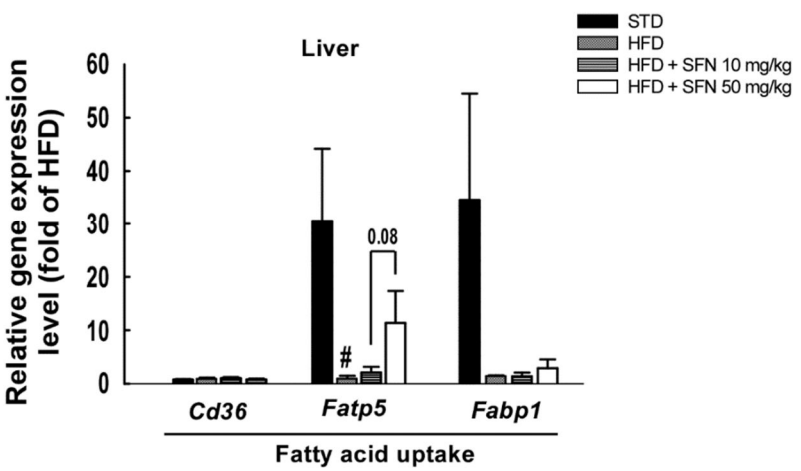
Figure 5. SFN increases the expression of mitochondrial oxidation-related proteins such as PGC1α, PPARα, and NRF1 in liver. SFN significantly increased mitochondrial oxidation-related proteins such as PGC1α (A, and B), PPARα (A, and C), NRF1 (A, and D). Administration of SFN (10 or 50 mg/kg) increased the mitochondrial biogenesis described as the ratio of mtDNA to nDNA in a dose dependent manner (E). "Black bars = CD + vehicle; gray bars =

HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01, $^{\#}$ P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

A


Liver
STD
HFD
HFD + SFN 10 mg/kg
HFD + SFN 50 mg/kg
Relative gene expression level (fold of HFD)
10.0
8.0
6.0
4.0
2.0
0
##
#
##
##
Pparγ
Srebp1c
Fasn
Scd-1
Dgat-1
fatty acid & triglyceride biosynthesis

B


Liver
STD
HFD
HFD + SFN 10 mg/kg
HFD + SFN 50 mg/kg
Relative gene expression level (fold of HFD)
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50
40
30
20
10
0
0.08
#
Cd36
Fatp5
Fabp1
Fatty acid uptake

C

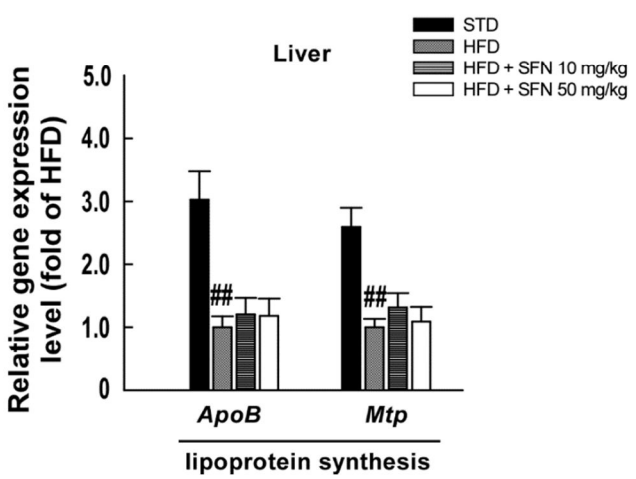
Liver
STD
HFD
HFD + SFN 10 mg/kg
HFD + SFN 50 mg/kg
Relative gene expression level (fold of HFD)
5.0
4.0
3.0
2.0
1.0
0
##
##
ApoB
Mtp
lipoprotein synthesis

Figure 6. SFN does not have effects on the expression of other lipid metabolic genes of liver. SFN did not alter not only fatty acid and triglycerides biosynthesis-related genes such as *Pparγ*, *Srebp1c*, *Fasn*, *Scd-1*, and *Dgat-1* (A) but also fatty acid uptake and transport-related genes such as *Cd36*, *Fatp5*, and *Fabp1* (B), cholesterol biosynthesis-related genes such as *ApoB*, and *Mtp* (C). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01, $^{\#}$ P<0.05 vs. CD group mice.

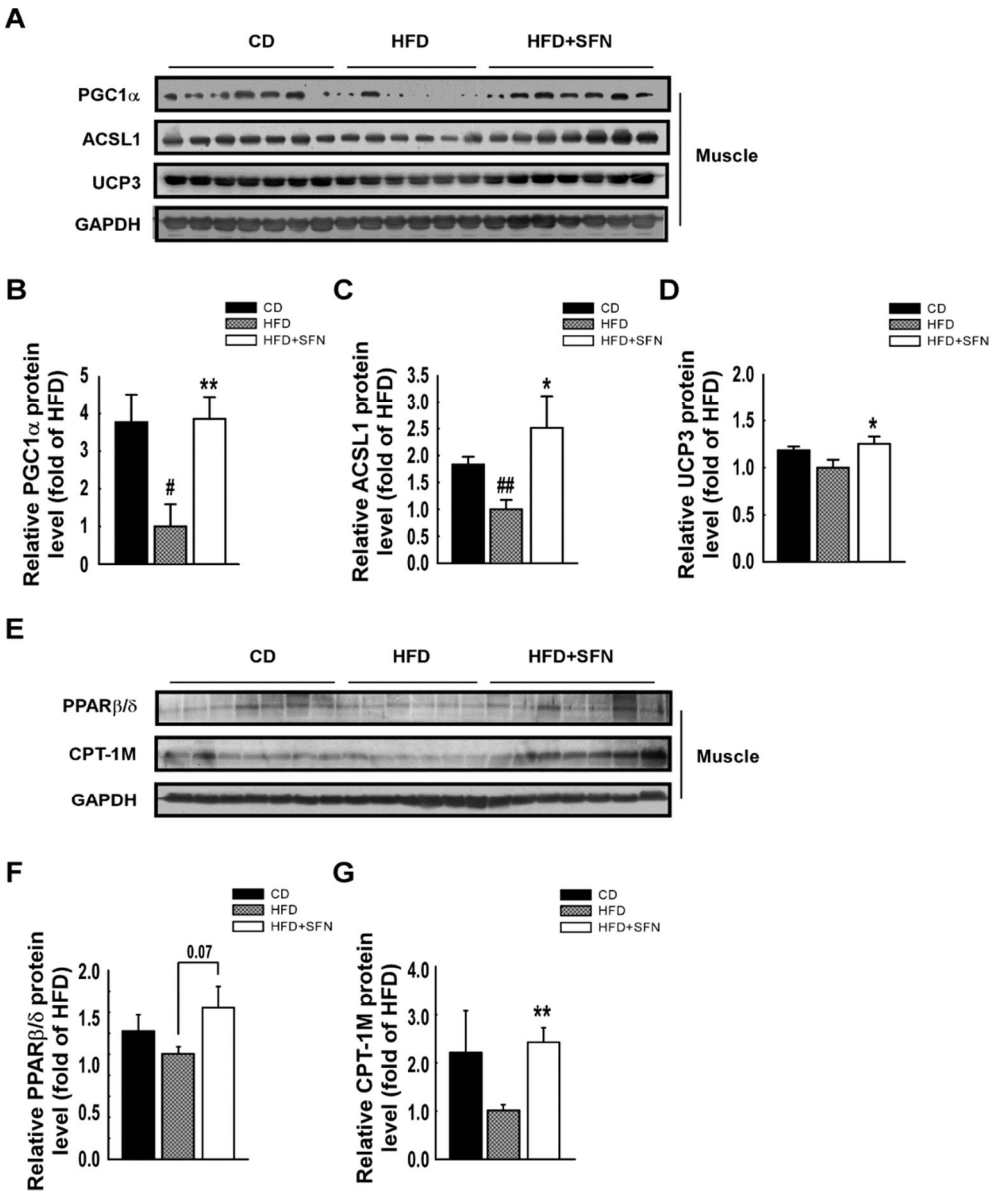

Figure 7. SFN up-regulates the expression of proteins related to fatty acid oxidation in skeletal muscle. SFN (50 mg/kg) treatment significantly increased the relative expression of fatty acid oxidation related proteins such as PGC1α (A, and B), ACSl1 (A, and C), UCP3 (A, and D), PPARβ/δ (E, and F), and CPT-1M (E and G) in skeletal muscle. "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg;

white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01, $^{\#}$ P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

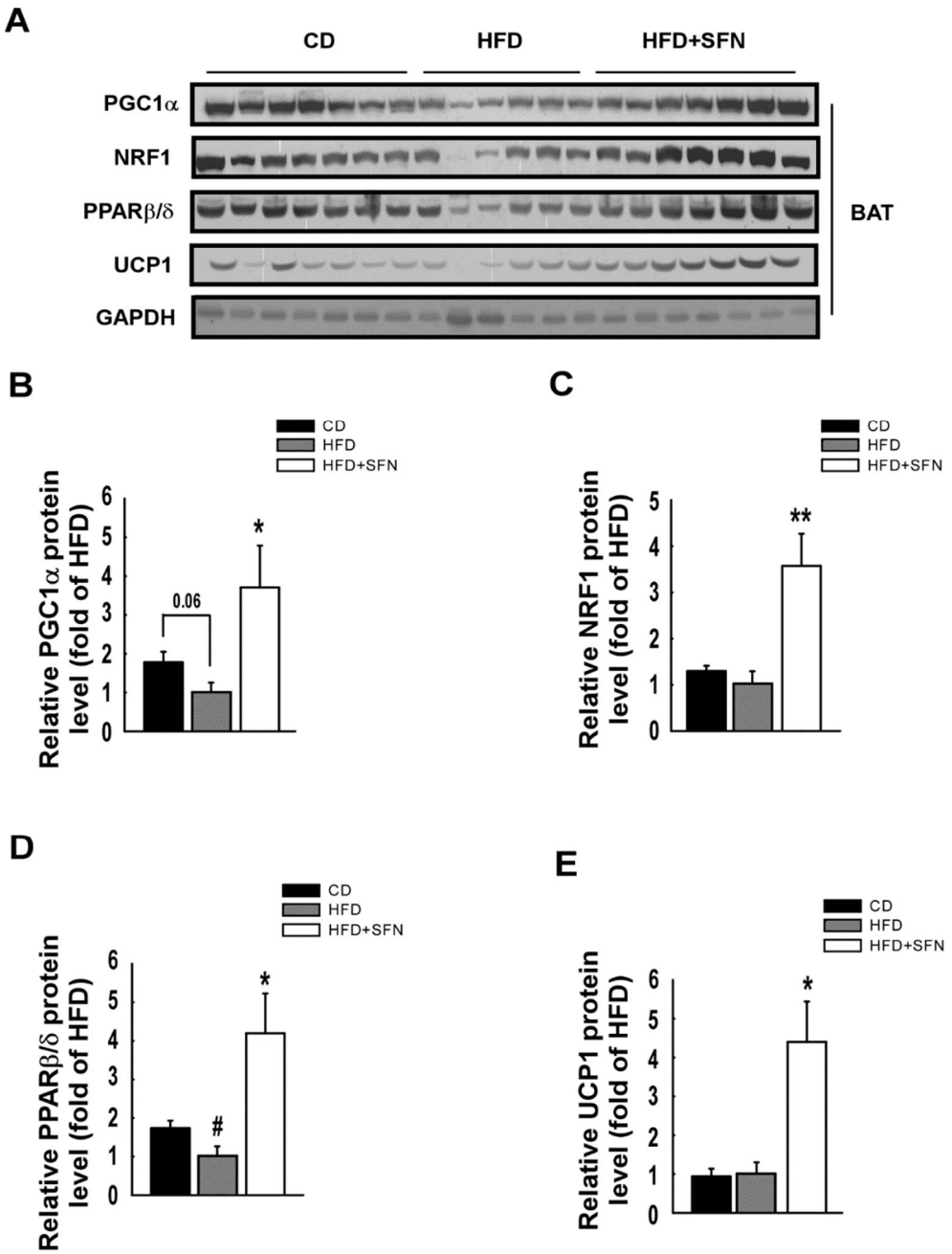

Figure 8. SFN increases the expression of proteins related to mitochondrial fatty acid oxidation (PGC1α, NRF1, PPARβ/δ), and thermogenesis (UCP1) in brown adipose tissue. Administration of SFN (50 mg/kg) significantly increased the protein expression of HFD-fed mice related to

mitochondrial fatty acid oxidation: PGC1α (A, and B), NRF1 (A, and C), and PPARβ/δ (A, and D). Moreover, SFN remarkably up-regulated the UCP1 expression (A, and E). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#}$ $P<0.05$ vs. CD group mice, $^{**}P<0.01$, $^{*}P<0.05$ vs. HFD group mice.

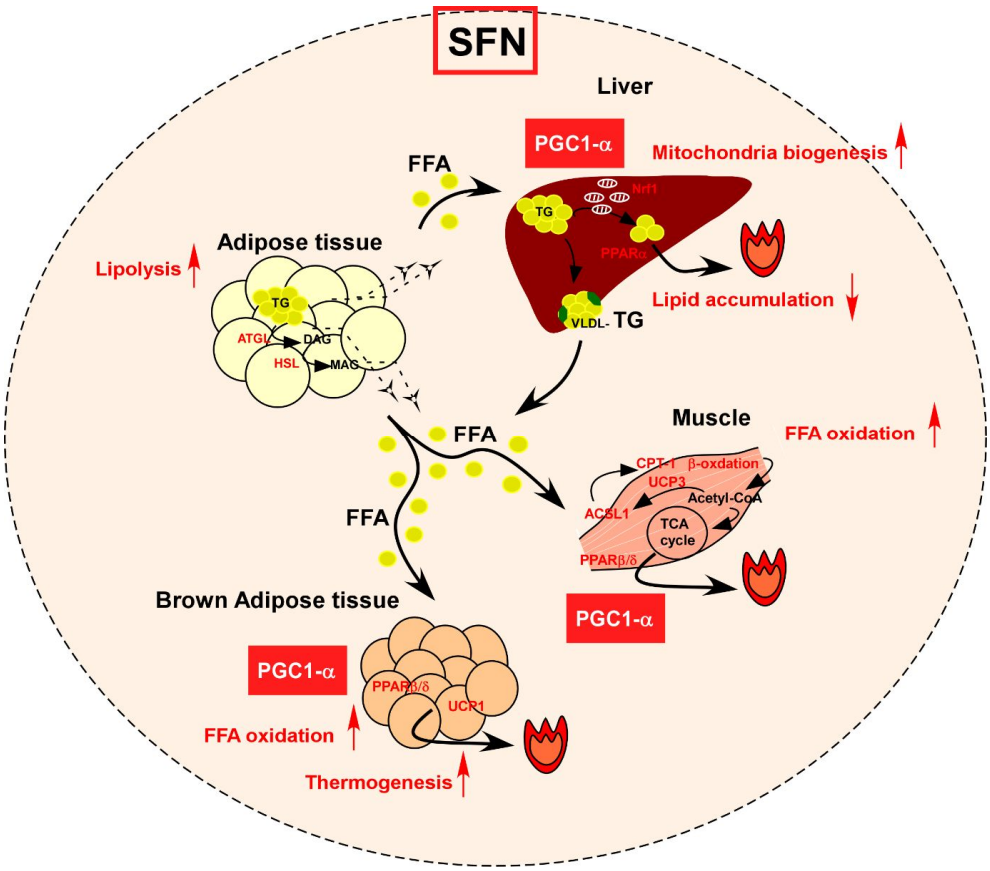

Figure 9. SFN enhances fatty acid metabolism in peripheral tissues. Summary. SFN promoted the lipase-mediated lipolysis in WAT simultaneously activating fatty acid oxidation in oxidative organs such as in the liver, muscle, and brown adipose tissue. Consequently, the lipid contents of WAT and liver were reduced by being used as a source of fatty acid oxidation in systemic oxidation metabolism in peripheral tissues.

Chapter 4

Sulforaphane regulates the transcription of PGC1α by inhibiting HDAC8 activity

Abstract

PGC1α, a key protein in energy metabolism, is regulated in multiple steps including the transcription, and post-translational modifications such as acetylation, phosphorylation. MEF2C, and CREB are major transcription factors of PGC1α. And besides of the transcription factors–mediated regulations, the epigenetic regulations such as DNA methylation by DNMT families, and histone modification by HDAC families also participate in the transcriptional regulation of PGC1α. Especially, recent findings provide evidences that the epigenetic alteration of PGC1α has the relevance in obesity, and obesity-related metabolic diseases such as type 2 diabetes (T2DM).

On the one hands, the HDAC inhibition-mediated increase of histone acetylation by SFN has been investigated from in cell to human studies in cancer. However, there's few study about the epigenetic regulatory effect of SFN in perspectives of obesity and obesity-related metabolic diseases. Moreover, even in cancer biology, there is a lack of studies about which certain isoform of HDACs has the preferences on the inhibition of SFN.

Here, I found that SFN increased the mRNA expression of PGC1α in major oxidative tissues including skeletal muscle, and liver. However, there was no significant changes in the expression of transcription factors such as MEF2C, and CREB. Thereafter, to examine epigenetic regulatory effect of SFN, I investigated whether SFN increased the histone acetylation by inhibiting HDAC activity in HFD-induced obesity mice model. As results, I found that HFD with SFN administration remarkably increased the global acetylation of histone H3, and

histone H4 in skeletal muscle compared to HFD feeding. Moreover, I found that SFN selectively inhibited HDAC8 activity, belonged in the class Ⅰ HDAC family, rather than other HDAC isoforms. Additionally, I revealed that the deletion of HDAC8 by lentiviral infection increased the expression of PGC1α in HeLa cell. Consequently, I concludes that SFN inhibits the HDAC8 activity, thereby, increase the transcription of PGC1α in HFD-induced obesity mice model. This mechanism may mainly contribute the anti-obesity effect of SFN.

Key words: *SFN, PGC1α, global histone acetylation, histone H3, histone H4, HDAC inhibition, HDAC8 activity*

4.1. Introduction

Peroxisome proliferator-activated receptor gamma co-activator-1α (PGC1α) is a powerful regulator modulating energy metabolic program. PGC1α regulates oxidative phosphorylation as well as contents of mitochondria (1, 2). PGC1α activation is controlled through various regulatory circuits including post-translational modification such as phosphorylation, acetylation, deacetylation, sumoylation, and methylation, in which various proteins such as AMP-activated protein kinase (AMPK), sirtuin1 (SIRT1), p38 mitogen-activated protein kinase (MAPK) were involved (3-7). In addition, PGC1α expression is also modulated by several transcription factors such as myocyte enhancing factor (MEF2C), cAMP response element-binding (CREB) depends on calcineurin A or calcium/calmodulin–dependent protein kinase (CaMK), respectively (8).

Besides of post-translational regulation or transcriptional regulation by transcription factors, it is well understood that epigenetic regulations such as DNA methylation or histone modification are also important regulatory mechanisms of PGC1α, which are mediated by DNA methyltransferase (DNMT) or histone deacetylase (HDAC) and histone acetyltransferase (HAT) (9, 10). For examples, the increase of methylation in PGC1α promoter occurred in T2DM patients and reduced the PGC1α expression compared to normal (10). Reversely, high-intensity exercise decreased the methylation in skeletal muscle-specific promoter of PGC1α controlling contraction of muscle (11). Additionally, it has been reported that trichostatin A (TSA) and valproic acid (VPA), as a representative HDAC inhibitors (HDACi) increased the PGC1α and PGC1α-related genes such as carnitine palmitoyltransferase 1 (CPT1) in neuroblastoma cells (12). Moreover, class Ⅱa

HDACs deacetylated MEF2 proteins suppressing MEF2-dependent transcription of PGC1α (9, 13). And pharmacokinetic specific inhibition of class Ⅰ HDACs showed good mitochondrial signature and promoted oxidative metabolism in skeletalmuscle, in which PGC1α involved (14). However, it is less revealed that which specific HDAC isoforms play important roles in epigenetic regulation of PGC1α expression in terms of controlling metabolic disease including obesity.

Recently, regulation of HDACs by dietary food component is suggested as a good strategy to control obesity (15). In chapter Ⅲ, I found that SFN increased PGC1α expression in oxidative peripheral tissues. However, intriguingly, recent previous studies demonstrate that SFN inhibits HDAC activity exerting anti-cancer effect (16, 17). But it is still unclear which isoform of HDACs is mainly involved in the action of SFN. Therefore, in this chapter, I examined how SFN regulates PGC1α expression considering SFN as a candidate for HDAC inhibitor. Consequently, although it is little known about the previous role of histone deacetylase 8 (HDAC8) in metabolism, I newly revealed that SFN increases the transcription level of PGC1α dependent HDAC8 using HDAC8 knockdown cell. Collectively, these results imply that the anti-obesity effect of SFN is explainable for histone modification of PGC1α by HDAC8.

4.2. Materials and Methods

4.2.1. Animals

C57BL/6J WT mice (Male, 6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed with free access to food and water in the animal facility of the Seoul National University in temperature-, light-, and humidity-controlled rooms with a 12-h light/dark cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-110126-8).

4.2.2. HFD-induced obesity and sample administration

Age-matched male mice were fed a standard diet (STD) (10 kcal% Fat, Research Diets, New Brunswick, NJ, USA) or chow diet (CD) as a control, or a HFD (60 kcal% Fat, Research Diets). Mice were divided into three or four groups (n=6-9 per group): (1) STD (or CD) + phosphate-buffered saline (PBS) control group, (2) HFD + PBS group, and (3-4) HFD + SFN (10 or 50 mg/kg) group. SFN (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in PBS and orally administrated every day for 8 weeks or 15 weeks.

4.2.3. Cell culture

HeLa cell line was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's

medium (DMEM) (Welgene, Korea) supplemented with 10% FBS (Sigma, St. Louis, MO, USA) and 1% Penicillin Streptomycin (Corning, Corning, NY, USA).

4.2.4. Lentiviral infection

Non-targeting shRNA (shCont), and shHDAC8 lentiviral particles were purchased from Santa Cruz (Dallas, TX, USA), 60% confluent HeLa cells were infected by each viral particles and incubated overnight. After incubation, cell culture medium was replaced with fresh DMEM for 24 hours. Selection of infected cells was done using puromycin (2 μg/mL) (Sigma, St. Louis, MO, USA) over 36 hours. The final selected HeLa cells were utilized for further experiments.

4.2.5. Western blot assay

Mouse tissues were harvested and homogenized in radio immunoprecipitation assay buffer (RIPA buffer) (Cell signaling, Danvers, MA, USA) with one tablet of protease inhibitor cocktail (Roche, Penzberg, Germany). After centrifugation (14000 rpm, 10 min), supernatant was collected and analyzed to determine the concentration of protein using protein assay kit (Bio-Rad, Hercules, CA, USA). Tissue lysates were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE healthcare, Piscataway, NJ, USA). The membrane was blocked with 5% skim milk and incubated with specific primary antibody followed by horseradish peroxidase (HRP)-conjugated secondary antibody. The protein bands

were visualized using a chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA). Antibodies against CREB (1:1000) were obtained from Cell Signaling (Beverly, MA, USA). Antibody against MEF2C (1:1000), HDAC8 (1:1000), and PGC1α (1:1000) were purchased from Abcam (Cambridge, MA, USA). Antibody against acetylated-histone H3 (1:1000) were attained from Millipore (Darmstadt, Germany). Antibody against histone H3 (1:1000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000), β-actin (1:5000) were obtained from Sigma (St. Louis, MO, USA). Band intensity was quantified with Image J

4.2.6. Quantitative real time poly chain reaction (qRT-PCR)

Mouse tissues were homogenized in RNA iso plus (Takara, Dalian, China) and total RNA was isolated using the RNA Mini kit (Ambion, Foster City, CA, USA). cDNA was synthesized by PrimeScript RTase (Takara, Dalian, China). Quantitative RT-PCR reaction was performed using CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). cDNA was amplified in the presence of SYBR Green (Bio-Rad). Relative expressions of mRNA level were calculated according to ddCt method (2^{-ddCt}) (18). The primer sequences were described in table 1.

Table 2. The primer sequences for qRT-PCR

Gene	F/R	Sequence
Ppargc1a (mouse)	Forward	5′-ATGGGCTGTGATCGGAACTG-3′

	Reverse	5′-GTCTTCCCAATAAGCATGTCTCC-3′
β-actin (mouse)	Forward	5'-TGTCCACCTTCCAGCAGATGT-3'
	Reverse	5'-AGCTCAGTAACAGTCCGCCTAGA-3'
Ppargc1a (human)	Forward	5’-GATGCGCTGACAGATGGAGA-3’
	Reverse	5’-TAGAGACGGCTCTTCTGCCT-3’
β-actin (human)	Forward	5’-TCCTCACCCTGAAGTACCCCAT-3’
	Reverse	5’-AGCCACACGCAGCTCATTGTA-3’

4.2.7. HDAC activity assay

In vitro HDAC array was conducted by epigenetics profiling service (Reaction Biology, Malvern, PA, USA). Briefly, each HDAC (1-11) enzymes were pre-incubated with SFN and then substrate mixture (fluorogenic HDAC Substrate and co-factor) was added and incubate 2 hr at 30°C to initiate the reactions. To stop the reaction, developer with Trichostatin A was added. After generating fluorescent color, measure the kinetics at Ex/Em 360/460 nm for 1.5 hr with 15 min interval.

4.2.8. Statistical Analysis

All data were expressed as the sample means ± S.E.M. Statistical mean differences between groups were verified using Student’s t-test. Probability values of $p < 0.05$ and 0.01 were used as criterion for statistical significance.

4.3. Result

4.3.1. SFN regulates PGC1α expression in transcriptional level in liver and skeletal muscle.

To elucidate how SFN regulated PGC1α expression, I confirmed whether SFN had effect on mRNA expression of PGC1α using qPCR. In liver and skeletal muscle, HFD fed group outstandingly decreased the mRNA expression level of PGC1α up to about four fold compared to STD fed group. And Consistently with PGC1α protein expression in chapter 3, SFN 50 mg/kg significantly increased PGC1α mRNA expression both in liver and skeletal muscle (Figure 1A and B). Although HFD only fed group also significantly reduced PGC1α mRNA expression in white adipose tissue, HFD+SFN (10 or 50 mg/kg) group did not altered PGC1α mRNA expression compared to HFD fed group, unlike liver and skeletal muscle (Figure 1C). On the one hand, while SFN increased PGC1α protein expression level (Chapter 3, Figure 9A), there were no changes in PGC1α mRNA expression in brown adipose tissue of all groups (Figure 1D). Overall, SFN regulates PGC1α expression in transcriptional level in liver and skeletal muscle. Since PGC1α-mediated fatty acid oxidation (FAO) in these highly oxidative tissues mainly contribute to energy expenditure of whole body (2), transcriptional regulation might be a main mechanism of SFN to regulate PGC1α expression in HFD fed mice. On other hand, apart from liver and skeletal muscle, it needs to be further investigated that SFN might regulate PGC1α expression via another pathway such as post-translational modification in brown adipose tissue, which could influent on thermogenesis.

4.3.2. SFN did not affect the expression of transcription factors of PGC1α, MEF2C, and CREB proteins in skeletal muscle.

Because SFN increased the transcription of PGC1α, I identified the expression of transcription factors of PGC1α in skeletal muscle in which showed most outstanding increase of PGC1α. MEF2C and CREB are representative transcription factors binding to promoter of PGC1α (2). I found that the protein expression of MEF2C was not changed in all groups (Figure 2A and B). Although HFD slightly increased the protein expression of CREB compared to CD, SFN (50 mg/kg) administration did not change the CREB expression compared to HFD (Figure 2A and C). These observations indicated that transcriptional regulation of PGC1α by SFN is not attributed to alteration of transcription factor expression level.

4.3.3. SFN increased global histone acetylation in Skeletal Muscle.

Besides of regulation by transcription factor such as MEF2C and CREB, PGC1α mRNA expression can be also controlled epigenetically (19, 20). Previous studies has been showed that SFN has an inhibitory effect on histone deacetylase (HDAC) activity resulting in some beneficial effects including anti-cancer effect (17, 21, 22). Thus, I investigated whether SFN affected the histone acetylation in skeletal muscle. HFD reduced the acetylation of histone H3 in skeletal muscle compared to CD. HFD + SFN (50 mg/kg) significantly recovered the acetylation of histone H3 reduced by HFD (Figure 3A-C). Consequently, SFN altered global

histone acetylation in skeletal muscle supporting that SFN had an effect on epigenetic regulation

4.3.4. SFN specifically suppresses HDAC8 activity.

Although some reports demonstrated that SFN inhibits total HDAC activity in various model (17, 23, 24), there are fewer evidences which specific isoform was affected by SFN. Thus, I conducted HDAC screening assay against HDAC isoforms (1-11) with SFN. The results showed that SFN only has specificity on HDAC8 enzyme belonged in class 1 HDAC having IC50 at 62.9 μM. On the other hand, SFN had no effect on other HDACs (Figure 4A). However, SFN did not reduce the expression of HDAC8 in skeletal muscle (Figure 5A and B). Therefore, these observations mean that SFN specifically inhibits HDAC8 enzyme activity resulting in epigenetic alteration.

4.3.5. SFN increases the expression of PGC1α dependent on HDAC8.

To further investigate whether the regulation of PGC1α by SFN depends on HDAC8 or not, I infected shRNA lentiviral particles into HeLa cells for deletion of HDAC8. Firstly, I compared HDAC8 HeLa cell with shCont HeLa cell against the expression of PGC1α to examine the relationship between PGC1α and HDAC8 proteins. HDAC8 expression was knockdown (KD) in shHDAC8 HeLa cell compared to shCont HeLa cell. Reversely, PGC1α expression was up-regulated in shHDAC8 HeLa cell compared to shCont HeLa cell (Figure 6A). Consistently with protein expression, knockdown of HDAC8 significantly increased the mRNA

expression level of PGC1α about two folds compared to control (Figure 6B). Additionally, I investigated whether increase of PGC1α expression by SFN depends on HDAC8. I found that SFN (250 nM) increased the protein expression of PGC1α in shCont HeLa cell (Figure 6C). However, SFN did not change the protein expression of PGC1α in absence of HDAC8 (Figure 6C). Overall, HDAC8 mediates the regulation of PGC1α expression by SFN.

4.4. Discussion

As growing incidences of epidemic obesity worldwide due to various environmental factors, for last decade, the relevance of epigenetics on obesity has been received attentions (25-27). Epigenetic regulations are typically reversible without any change on DNA sequence itself and generally include DNA methylation, post-translational modification of histone proteins such as acetylation, deacetylation, methylation, and modification by small non-coding RNA (miRNA) (28).

Many previous studies are focused on DNA methylation as an epigenetic marker. Some previous studies demonstrated that the addition of methyl groups on certain CpG sites (methylation) in several metabolic genes such as PGC1α is associated with the risk of obesity (29, 30). The level of methylation is different between groups with or without intervention for weight loss (31, 32). Nevertheless, summarized on most recent findings about the relationship between DNA methylation and obesity, systemic overview concluded that there is little consistent evidences for the relationship between global DNA methylation and obesity (20).

Although there are relatively few studies about the relationship between histone modification and obesity, previous researches have showed that global histone modification associated with obesity (33, 34). In details, the acetylation or deacetylation of histone proteins by HAT or HDAC enzyme in several metabolic genes changes compactness of chromatin activating or repressing the expression of metabolic genes and appeared differently between normal state and disease-state like obesity (35, 36). Recently, it is reported that several natural food

components such as curcumin, resveratrol, and epigallocatechin 3-gallate (EGCG) as well as diets, and nutrients act as a epigenetic modulator resulting in beneficial effects on metabolic system (15, 37). Especially, it is recently well understood that SFN acts as an inhibitor of histone deacetylase resulting in anti-cancer effects both *in vitro* and *in vivo* (16, 17, 38). Most of all, the inhibitory effect of SFN on HDAC activity is available even in the lower concentration of SFN compared to apoptosis and oxidative stress occurred in higher concentration of SFN *in vitro* (39). Thus, it is highlighted that epigenetic regulation of SFN might be more sensitive and primary mechanism than other mechanism of SFN. However, by so far, it is unclear that whether SFN has a beneficial effect on obesity and metabolic disorders through inhibitory activity of HDAC.

Total mammalian classical HDACs are divided into 4 classes; class Ⅰ HDACs (HDACs 1, 2, 3, and 8), class Ⅱ HDACs (HDACs 4, 5, 6, 7, 9, and 10), class Ⅳ HDACs (HDAC 11) as their characteristics (40). Recently, previous study demonstrate that specific inhibitor of class Ⅰ HDACs but not class Ⅱ HDACs induce mitochondrial oxidative metabolism in skeletal muscle and adipose tissue supporting that inhibition of class Ⅰ HDAC is more important to regulate energy metabolism (14). Although they suggested that HDAC3 belonged in class Ⅰ HDACs is one of major contributors, deletion of HDAC3 showed partially mimic effect to the effect of class Ⅰ HDACs specific inhibitor. Thus, the role of other HDACs belonged in class Ⅰ HDACs including HDAC8 is required to be further explored.

In this study, for the first time I found that HDAC8 as a specific novel

target of SFN, regulates PGC1α. HDAC8 is involved in class Ⅰ HDACs and is well known as a major epigenetic modulator in cancer, and other diseases (41). Moreover, HDAC8 specific inhibitor is received interests as an anti-cancer agent in clinical status (42). Here, I found that SFN, a natural compound from broccoli sprouts, inhibited specifically HDAC8 activity and sequentially it might induce histone acetylation and transcription of PGC1α (Figure 7).

I found that SFN inhibit HDAC8 activity at IC50 62.9 μM *in vitro*. But it seems to be too high to have physiological relevance. However, it is possible to assume that in vitro HDAC enzyme activity screening system was deficient for physiological environmental co-factors including metal ions such as Co(Ⅱ) or Zn(Ⅱ), and monovalent cations such as Na+ or K+. Substrate binding affinity of HDAC8 is greatly influenced depends on the concentration of metal ions, and monovalent cations, which catalyzed HDAC8 activation (43, 44). Thus, binding affinity of SFN on HDAC8 enzyme might be higher in co-factor enrich physiological conditions. Secondly, previous study demonstrated that SFN usually metabolized to several metabolites such as SFN-glutathione-s-transferase (GSH), SFN-cysteine (Cys), and SFN-n-acetyl-l-cysteine (NAC). And they had higher binding affinity to HDAC enzyme than parent compound, SFN. It is because that their characteristics of structures is more favorable to bind with HDAC enzymes (39). Thus, SFN might more effectively inhibits HDAC8 activity in physiological body with synergistic effects of its metabolites.

On the one hands, unlikely other HDACs in class Ⅰ HDACs, HDAC8 also has non-histone substrates such as estrogen-related receptor α (ERRα), CREB

besides of histone proteins (45). Previous researches demonstrated that overexpression of HDAC8 reduces the phosphorylation of CREB and its activity suggesting that HDAC8 interact with CREB proteins, which is one of the transcription factor of PGC1α (46). Therefore, despite I already confirmed that expression of CREB was not changed in Figure 2, it is possible that SFN has also indirect mechanism through increase of CREB activity by inhibition of HDAC8.

And it is necessary to interpret the observations found in this study more carefully, because it is not clearly defined yet histone deacetylase activity of HDAC8 between *in vitro* and *in vivo* because there are controversies (41). Consequently, I identified that SFN regulates the transcriptional regulation of PGC1α by inhibiting HDAC8 activity using HDAC8 KD cell model, but it needs to be further explored to direct or indirect mechanism of SFN's overall action.

4.5. References

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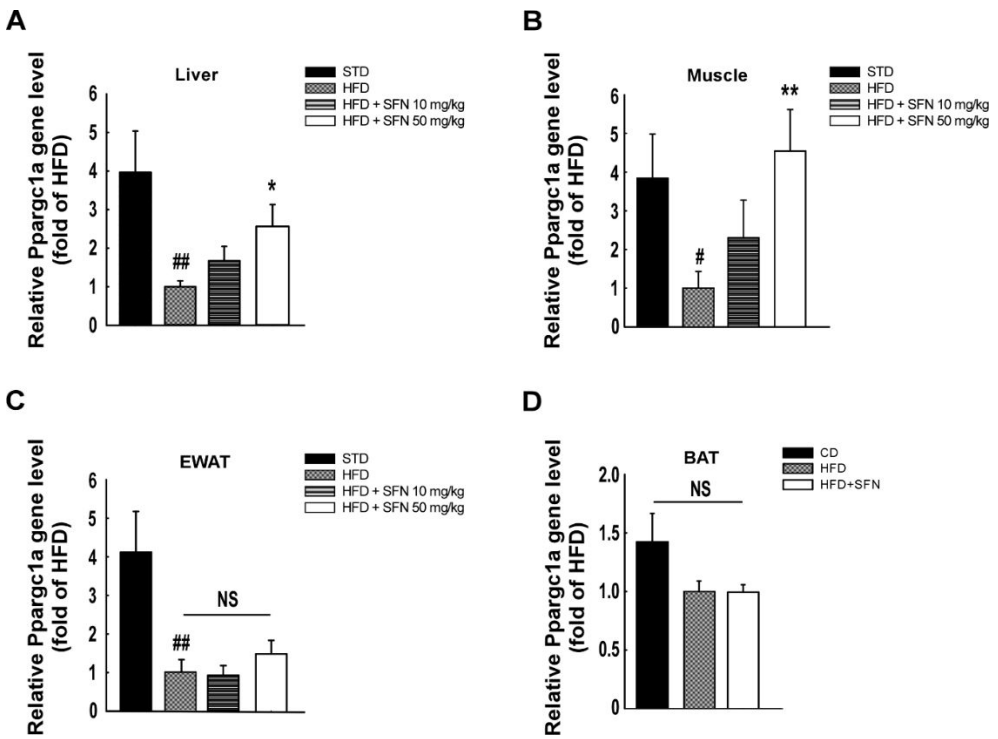

Figure 1. SFN increases the mRNA expression of PGC1α in liver and skeletal muscle. SFN significantly up-regulated the relative *Ppargc1a* gene expression in liver (A) and muscle (B). However, SFN did not change the expression of *Ppargc1a* gene reduced by HFD in epididymal white adipose tissue (C). There were no significant differences among all groups in brown adipose tissue (D) "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01, $^{\#}$ P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

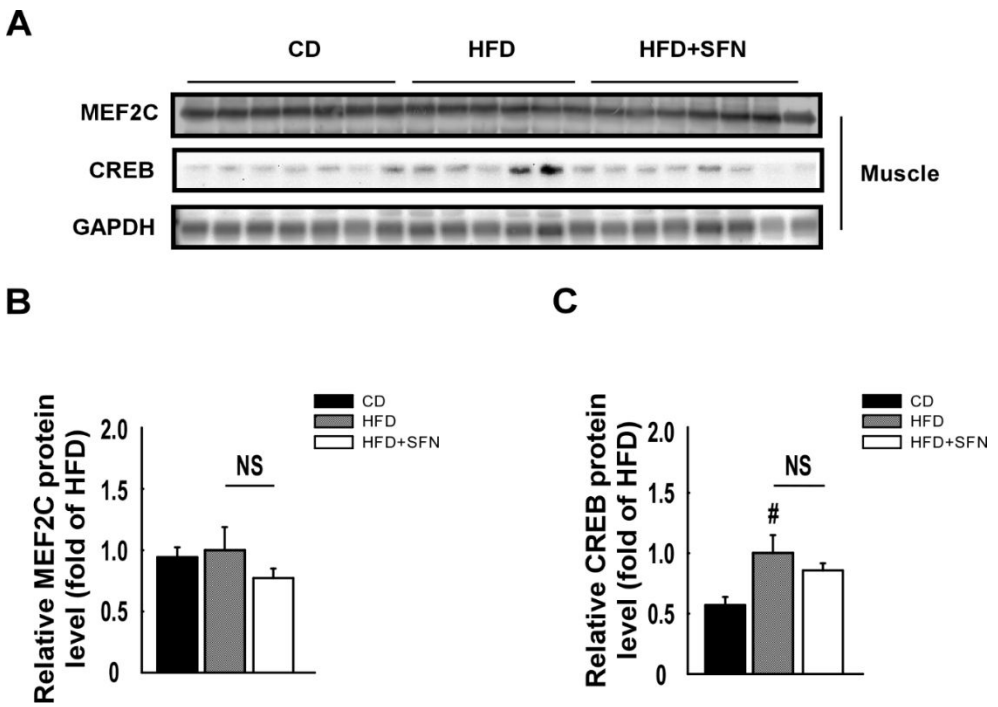

Figure 2. SFN does not alter the expression of transcription factor of PGC1α (MEF2C and CREB) in skeletal muscle. SFN (50 mg/kg) treatment did not increase the expression of MEF2C protein (A, and B). While HFD increased the expression of CREB protein, administration of SFN (50 mg/kg) did not change the expression (A, and C). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. # $P<0.05$ vs. CD group mice.

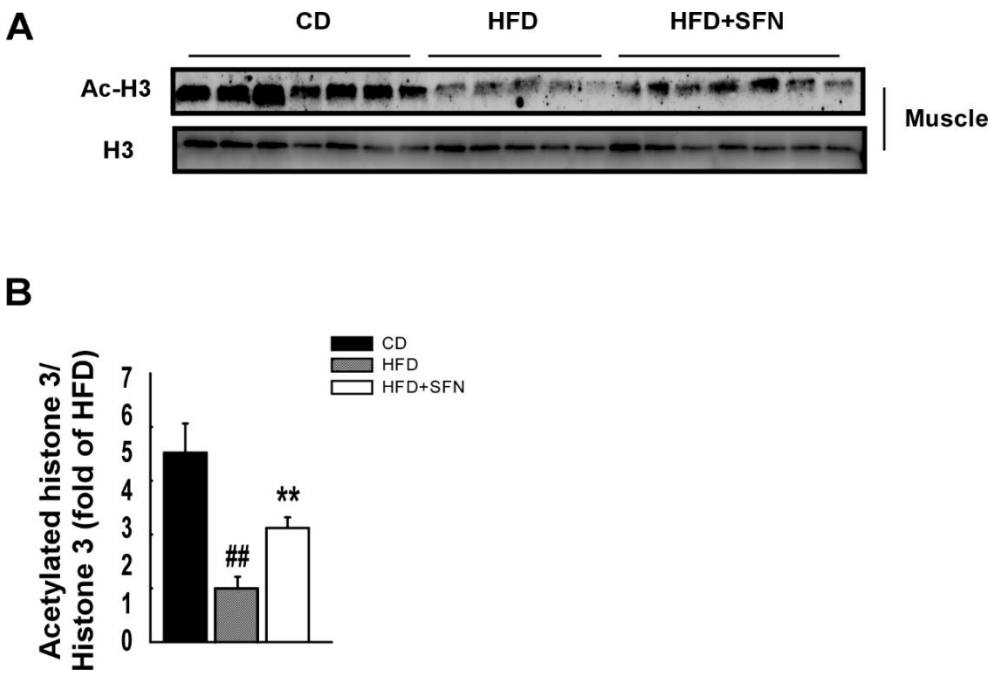

Figure 3. SFN acetylates histone H3 and H4 IN skeletal muscle. SFN (50 mg/kg) increased the acetylation of histone H3 (A and B) normalized with histone H3. "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars with horizontal stripe line = HFD + SFN 10 mg/kg; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01, $^{\#}$ P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

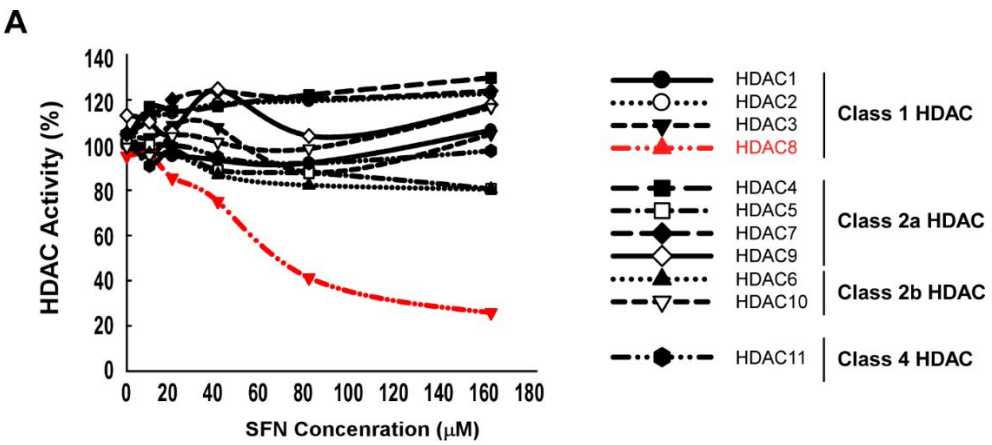

Figure 4. SFN specifically inhibits HDAC8 activity. Among 11 classical HDACs, SFN inhibited the activity of HDAC8 but not others. IC50 of SFN against HDAC8 activity was 62.9 μM (A). Data represents the mean ± SD. "Black circles and straight line = HDAC1; white circles and dotted line = HDAC2; black triangles and dashed line = HDAC3; red triangles and broken line = HDAC8; black squares and dashed line = HDAC4; white squares and broken line = HDAC5; black diamonds and dashed line = HDAC7; white diamonds and straight line = HDAC9; black triangles and dotted line = HDAC6; white triangles and dashed line = HDAC10; black circles and broken line = HDAC11." SFN (50 mg/kg) treatment reduces HFD-increased the HDAC8 activity in skeletal muscle (B). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. ## P<0.01, # P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

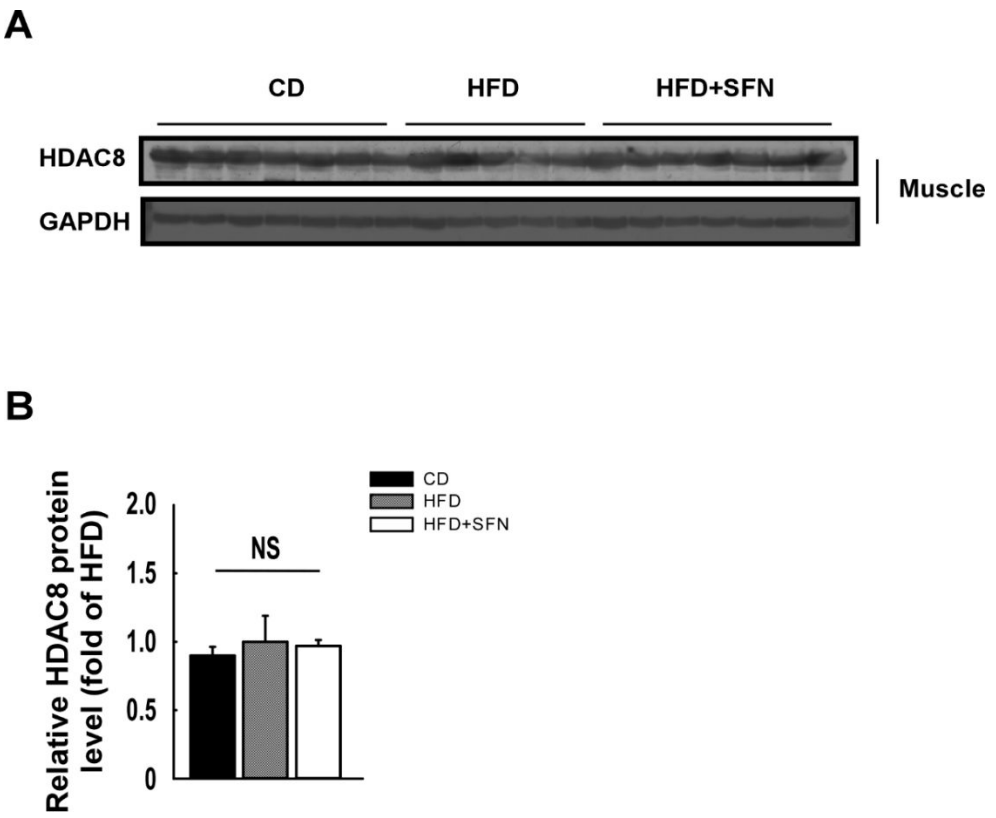

Figure 5. SFN does not decrease the expression of HDAC8 expression in skeletal muscle of HFD-fed mice. There were no significant differences against HDAC8 expression among all three groups, CD, HFD, and HFD+SFN (50 mg/kg). "Black bars = CD + vehicle; gray bars = HFD + vehicle; white bars = HFD + SFN 50 mg/kg." Data represents the mean ± SEM. $^{\#\#}$ P<0.01, $^{\#}$ P<0.05 vs. CD group mice, **P<0.01, *P<0.05 vs. HFD group mice.

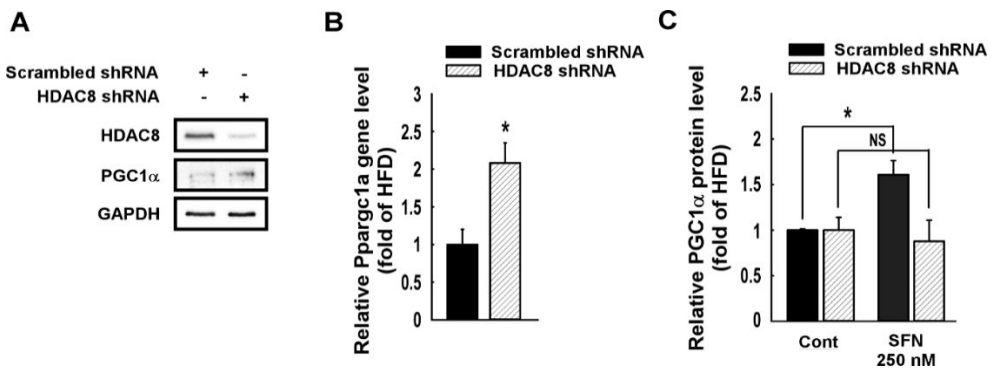

Figure 6. HDAC8 is involved in SFN-mediated increase in PGC1α expression. Knockdown of HDAC8 by infection of shRNA lenti viral particles induced an increase in PGC1α protein expression compared to control infected with non-selected shRNA (A). Ppargc1a gene expression was also increased in HDAC8 knockdown HeLa cell consistent to protein expression (B). While even 250 nM SFN treatment increased the mRNA expression of PGC1α, this effect was disappeared when HDAC8 expression was knockdown (C). "Black bars = Cont shRNA; white bars with diagonal line = HDAC8 shRNA." Data represents the mean ± SEM. *P<0.05 vs.shCont HeLa cell.

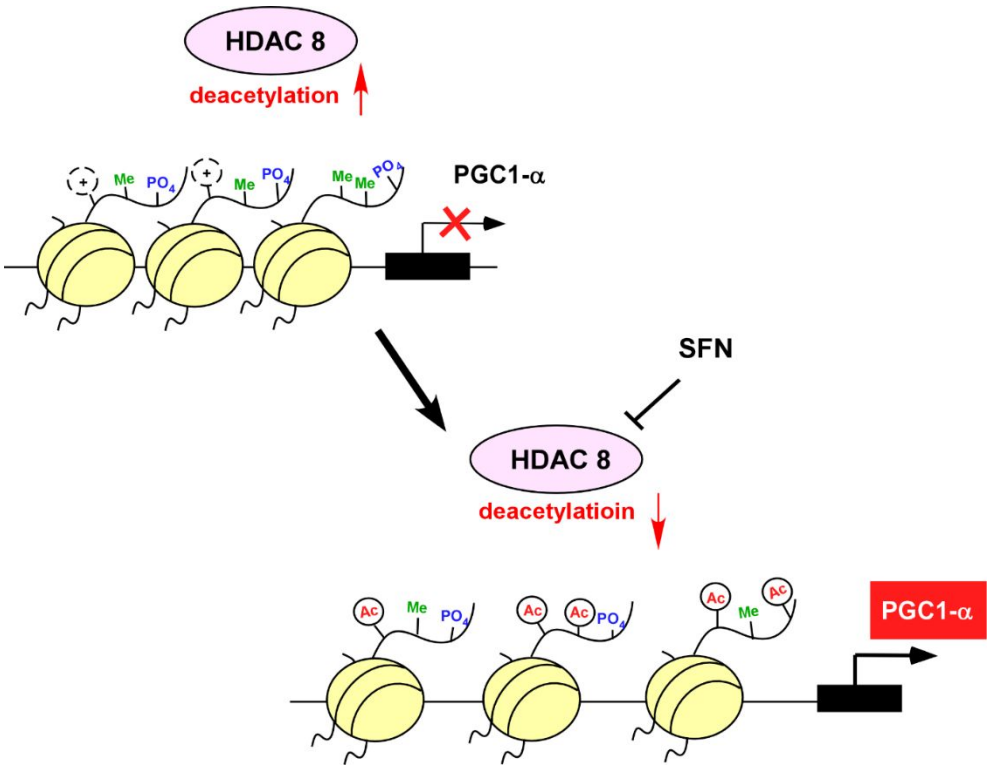

Figure 7. SFN regulates transcription of PGC1α by inhibiting HDAC8-medated histone deacetylation. Summary. SFN suppressed the deacetylation of histone proteins by inhibiting HDAC8 activity accelerating PGC1α transcription.

Chapter 5

Conclusion

5.1. Conclusion

In this study, I shed new light on the role of SFN as a natural epigenetic regulator in obesity beyond cancer biology. Moreover, for the first time, I suggested HDAC8 as a selective novel target of SFN to regulate PGC1α, a key protein in energy homeostasis.

Summarized in chapter 1, I reviewed the obesogenic environment factors as complex causes of the development of obesity, thereby, highlighted the new insight about the importance of epigenetic-based therapy in obesity intervention. Most of all, I emphasized that the intake of dietary foods with epigenetic regulatory effect in daily life or dietary food-driven natural epigenetic regulator including sulforaphane (SFN) could be easily affordable strategies for controlling obesity especially in terms of safety, and sustainability..

Summarized in chapter 2, I investigated that the role of Nrf2 in anti-obesity effect of SFN using genetically Nrf2 deleted mice. Opposite from the previous believes that most beneficial effects of SFN on various diseases were primarily due to Nrf2, anti-obesity effect of SFN was not depends on Nrf2 system. These observations indicate that Nrf2 is not involved in the anti-obesity effect of SFN, and other mechanism will play a major role in SFN's action.

Summarized in chapter 3, thereafter, I examined the effect of SFN on lipid metabolism in various peripheral tissues such as white adipose tissue (WAT), liver, skeletal muscle, and brown adipose tissue (BAT). As a results, SFN up-regulated the expression of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in WAT, thereby, increased the level of circulating fatty acids and glycerol in

serum. Apart from WAT, SFN promoted peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α)-mediated fatty acid metabolism-related proteins expression. In liver, skeletal muscle, and BAT. In liver, SFN induced the mitochondrial biogenesis increasing the expression of nuclear respiratory factor 1 (NRF1). Mitochondrial oxidation-related proteins such as acyl CoA synthetase 1 (ACSL1), carnitine palmitoyltransferase I (CPT1), peroxisome proliferator-activated receptorβ/δ (PPARβ/δ) were up-regulated by SFN in skeletal muscle. In BAT, SFN elevated the thermogenesis-related protein, uncoupling protein 1 (UCP1) as well as NRF1, and PPARβ/δ. Collectively, SFN enhance the PGC1α-mediated lipid metabolism in each peripheral tissues resulting in reducing WAT weight, which is significantly correlated with reduction of body weight.

Finally, summarized in Chapter 4, I further investigate the regulatory mechanism of PGC1α by SFN for comprehensive understanding of SFN's action in high fat diet (HFD)-fed mice. SFN remarkably increased the PGC1α expression in transcriptional level especially in liver and skeletal muscle. While the expression of PGC1α transcription factors such as myocyte enhancer factor 2C (MEF2C), and cAMP-response element binding protein (CREB) did not change, global acetylation on histone H3, and histone H4 were increased by SFN. With screening of SFN's inhibitory effect against total 11 classical histone deacetylases (HDACs), I revealed that HDAC8, one of class Ⅰ type HDACs, is a novel, and selective target for SFN among HDACs families. Moreover, the relevance between HDAC8 and PGC1α is also uncovered in HDAC8 knockdown HeLa cell. Overall, I demonstrates that SFN regulated PGC1α, as a master regulator of energy homeostasis, in transcriptional level by selectively inhibiting HDAC8 activity.

Consequently, I conclude that SFN suppresses HFD-induced obesity and insulin resistance by regulating PGC1α-mediated mitochondrial biogenesis, oxidation, and thermogenesis in various peripheral tissues such as WAT, liver, skeletal muscle, BAT through selective inhibition of HDAC8 (Figure 1).

These findings provide evidences supporting obesity intervention with natural epigenetic regulator such as SFN can be helpful and expected effective results. Therefore, it is required to discover various dietary food-driven natural epigenetic regulators besides of SFN. However, although epigenetics already has been widely studied in cancer biology, there is still limitation to application because most epigenetic regulators are “pan-inhibitors”, which target multiple isoforms, thereby, occurs unexpected side effects. Therefore, further investigation is needed to realize the next-generative epigenetic-therapy in personalized obesity intervention.

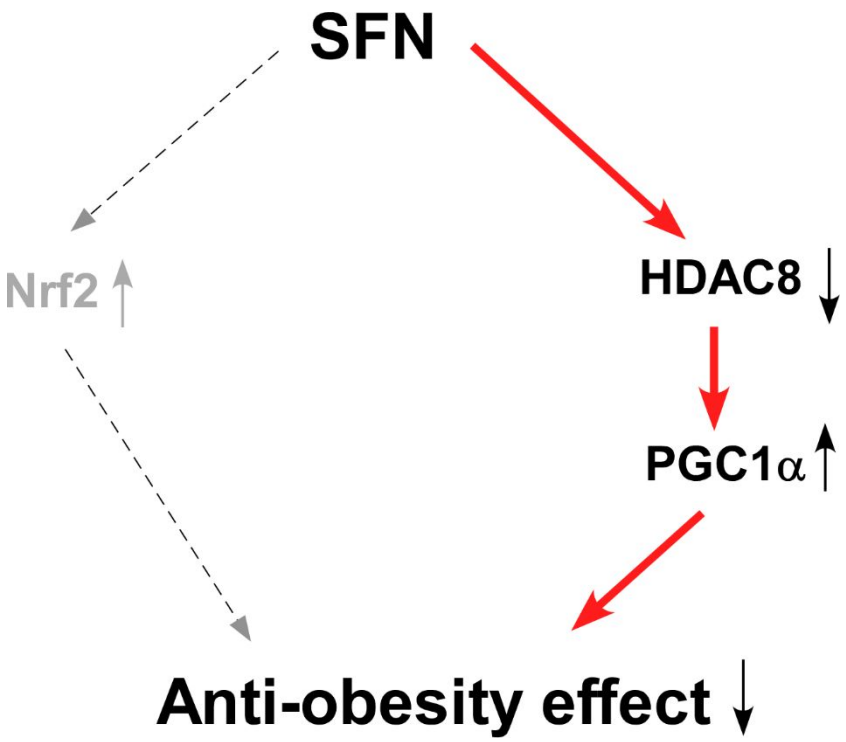

Figure 1. The novel underlying mechanism of anti-obesity effect of SFN in HFD-fed mice. SFN inhibited HDAC8 activity resulting in promotion of PGC1α transcription in different way from Nrf2 mechanism. This is a novel mechanism, which mainly contributes the anti-obesity effect of SFN in HFD-fed mice.

국문 초록

전세계적으로 비만 발병률이 증가함에 따라 비만으로 인한 제2형 당뇨, 고혈압, 심혈관 질환 등 다양한 대사성질환의 위험이 높아지고 있다. 이러한 비만의 원인은 단순하지 않고, 식이, 앉아있는 시간, 스트레스 등 복잡 다양한 환경 요인들에 의해 비만 및 비만 관련 대사성질환이 야기될 수 있다고 알려져 있다. 이와 같이 우리 주변에는 비만을 야기할 수 있는 환경 요인들이 늘 존재하기 때문에, 효과적이고 장기적인 비만 예방 및 치료 전략에 대한 필요성이 요구된다.

비만을 중재하는 방법으로는 생활 습관 개선, 비만 치료 수술, 비만치료제를 활용한 약물치료 등이 있다. 그런데 이 중 수술 또는 약물치료와 같이 일부 접근 방법들에 대해서는 부작용과 같은 안전성에 대한 우려나 장기적으로 지속할 수 없다는 점에서 한계점이 존재한다. 이러한 맥락에서, 식품은 오랫동안 먹어온 경험에 따라 안전성이 검증되고, 일상생활에서 지속적으로 섭취가 가능하다는 면에서 많은 이점이 있다고 할 수 있다. 최근 연구결과들에 따르면, 우리 주변에 존재하는 비만을 야기

할 수 있는 다양한 환경 요인들 뿐 아니라 우리가 일상적으로 섭취하는 다양한 몇몇 식품들에 의해서도 후생학적 유전적인 변화를 일으킬 수 있다고 보고되고 있다. 따라서 후생유전학적 관점에서 천연 조절성분들에 의한 약물치료 또는 해당 성분이 풍부한 식품을 매일 섭취하는 것이 비만을 예방 또는 치료하는데 있어 새로운 전략으로써 제시될 수 있다.

설포라판은 매일 접할 수 있는 브로콜리와 같은 십자화과 식물들에 풍부하게 들어있는 천연성분이다. 많은 연구들이 비만, 당뇨를 포함하는 다양한 질환들에서 설포라판이 이로운 효과들을 가진다고 보고하고 있다. 일반적으로 이러한 설포라판의 효과들은 대부분 Nrf2 단백질의 활성을 통해 나타나는 것으로 알려져 있어, 다양한 논문들에서 SFN을 Nrf2 활성화 작용제로서 빈번하게 사용하고 있다. 그러나 대사성질환에서는 Nrf2의 상반된 역할에 대한 연구결과들이 보고됨에 따라 비만 및 제 2형 당뇨 등 비만 관련 대사성 질환에서의 설포라판의 이로운 효과에 있어 Nrf2의 역할에 대한 정확한 연구가 요구된다고 할 수 있다.

최근 설포라판은 특히 암 생물학 분야에서 Nrf2 기작과 별개로써 천연 HDAC 저해제로서 주목 받고 있다. 그러나 HDAC

저해제로서의 SFN에 대한 역할은 비만 및 관련 대사성질환에서는 거의 연구된 바가 없다. 더욱이 암 생물학 분야에서 조차, 설포라판이 어느 특정 HDAC에 대해 우선성을 가질지에 대해서는 불분명한 상태이다.

본 연구에서는 Nrf2 KO 쥐를 사용하여 설포라판의 항비만 효과에 대한 Nrf2 의존성을 연구하고자 하였다. 그 결과, 설포라판은 Nrf2-WT과 Nrf2-KO 쥐 모두에서 체중 증가 억제 및 인슐린 감수성을 개선하는 효과를 나타냄을 관찰할 수 있었다. 또한 이러한 감소는 식이 섭취 및 지방 배출 변화에 따른 결과가 아님을 알 수 있었다. 설포라판의 비만 억제 기작을 추가적으로 연구하기 위해 고지방식이를 먹인 쥐의 백색지방, 간, 근육, 갈색 지방과 같은 다양한 말초조직에서의 지방대사에 미치는 설포라판의 효과를 알아보고자 하였다. 그 결과, 설포라판은 백색지방에서 ATGL, HSL과 같은 지방분해효소의 발현을 증가시킴으로써 백색지방의 무게를 감소시키고 혈중 지방산 농도를 올리는 것을 확인하였다. 또한, 설포라판은 에너지 생성을 위해 지방산 소비 기작을 촉진시키는 것을 확인하였는데, 자세하게는 각 조직에서 에너지 대사에서 주요한 역할을 하는 PGC1α 단백질 발현 증가와 함께

간에서 NRF1과 같은 미토콘드리아 생성 관련 단백질들의 발현 증가 및 근육에서 PPAR 단백질들을 포함하는 미토콘드리아 지방산 연소 관련 단백질들의 발현 증가, 갈색지방에서 UCP1과 같은 열발생을 통한 지방산 연소 관련 단백질의 발현이 증가함을 확인하였다. SFN이 이러한 PGC1α 를 어떻게 조절하는지 연구한 결과, 설포라판은 전사 과정에서부터 PGC1α의 발현을 증가시키는 것을 알 수 있었고, MEF2C나 CREB과 같은 전사인자들의 변화에는 설포라판이 영향을 미치지 않음을 알 수 있었다. 그러나, 설포라판이 근육 조직에서 히스톤 단백질 H3, H4에서의 전체 아세틸레이션 정도를 증가시키는 것을 관찰함에 따라 전형적인 HDAC들 중 SFN의 효과를 설명할 수 있는 특정 HDAC을 조사하기 위해 11개의 HDAC 효소 저해 여부를 스크리닝하였다. 그 결과, SFN은 선택적으로 HDAC8의 활성을 저해함을 알 수 있었다. 반면, 설포라판은 근육 조직에서 HDAC8의 발현 변화에는 영향을 주지 않았다. HDAC8과 PGC1α 간의 상대적 관계에 대해 밝히기 위해 렌티 바이러스를 활용한 HDAC8이 제거된 HeLa 세포를 만들어 사용하였으며, 이를 통해 HDAC8이 제거됨에 따라 PGC1α의 발현 증가가 나타남을 알

수 있었고, 설포라판에 의해 PGC1α 발현이 증가하는 효과는 HDAC8이 제거된 세포에서는 나타나지 않는 것을 관찰 함에 따라 설포라판의 PGC1α 조절 기작은 HDAC8에 의존적임을 알 수 있었다.

종합적으로, 본 연구에서는 처음으로 설포라판이 Nrf2와는 비의존적인 작용 기전으로서 기존 관념과는 달리 새로운 타겟인 HDAC8 저해를 통해 각 말초 조직에서 PGC1α로 매개되는 지방대사를 조절함으로써 고지방식이로 유도된 비만을 억제하는 효과를 가짐을 밝혔다. 이러한 연구 결과는 일상생활에서의 지속적인 브로콜리 섭취를 통해서나 천연후성조절자로서의 설포라판 단일 성분을 활용한 새로운 약물 치료 등의 방법으로 장기적으로 생애에 걸쳐 후생학적 조절을 통한 비만을 중재를 할 수 있는 근거를 제시하였다는 점에서 의의를 가진다고 할 수 있다.

검색어: 비만, 대사성질환, 설포라판, 엔알에프투 (Nrf2), 히스톤 디아세틸레이즈 저해제 (HDACi), 후생학적 조절, 지방 대사, 피쥐씨원 알파 (PGC1α), 히스톤 디아세틸레이즈 8 (HDAC8)